The role of bone morphogenetic proteins in normal and malignant lymphocytes

by

Kanutte Huse

Department of Immunology

Institute for Cancer Research

The Norwegian Radium Hospital

Oslo University Hospital

Faculty Division the Norwegian Radium Hospital

Faculty of Medicine

University of Oslo

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Kanutte Huse
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Errata

Location	Submitted version	Corrected version
p6	unbiquitin	ubiquitin
p6, p16	XBP1	XBP-1
p11	T17	Th17
p12	interact	interacts
p12	mediate	mediates
p14, p34, p46	germinal centre	germinal center
p15	cell	cells
p15, p16, p39	Blimp1	Blimp-1
p16, s18	post GC	post-GC
p18	Ig-translocation	Ig translocation
p18	traslocation	translocation
p19	CG B cell	GC B cell
p19	Ig-loci	Ig loci
p21, p22	Ig-genes	Ig genes
p21, p25	trough	through
p23	Haematopoietic	Hematopoietic
p23	lead	leads
p23, p25, p26, p29, p41	signalling	signaling
p24	interact	interacts
p25	sigaling	signaling
p26	carciogenesis	carcinogenesis
p30	were	was
p32	3H-thymidin	3H-thymidine
p32	studies	studied
p32	lymophoma	lymphoma
p32	over-expression	overexpression
p34	form	from
p34	cell lines	cell line
p35	Ig secreting	Ig-secreting
p36	dependent of	dependent on
p38	remains	remain
p38	over-expressed	overexpressed
p40	induced	induce
p40	types	type
p41	tumour	tumor
p41	recently	recent
p41	a EBV-negative	an EBV-negative
p42	singling	signaling
p43	suggests	suggest
p44	showed	shown
Figure 5	Follicular lympoma	Follicular lymphoma
Figure 5	NK-κB	NF-κB

Contents

Abbreviations

ASHM	Aberrant somatic hypermutation
ACTRII	Activin receptor II
AID	Activation-induced deaminase
Alk	Activin receptor-like kinase
APC	Antigen-presenting cells
BCR	B cell receptor
BL	Burkitt lymphoma
Blimp-1	B-lymphocyte-induced maturation protein 1
BMP	Bone morphogenetic protein
BMPRII	BMP receptor II
CSR	Class switch recombination
D-region	Diversity region
DLBCL	Diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
EGR1	Early growth response 1
FDC	Follicular dendritic cell
FL	Follicular lymphoma
GC	Germinal center
Ig	Immunoglobulin
IgH	Ig heavy chain
IgL	Ig light chain
IRF4	Interferon regulatory factor 4
iTreg	Inducible regulatory T cell
J-region	Joining region
NHL	Non-Hodgkin lymphoma
MHC	Major histocompatibility complex
MiR	MicroRNA
R-Smad	Receptor-regulated Smad
SHM	Somatic hypermutation
Smurf1	Smad ubiquitin regulatory factor 1
TAB1	TAK1 binding protein 1
TAK1	TGF-β activated kinase 1
TCR	T-cell receptor
Th	Helper T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
V-region	Variable region
XBP-1	X-box binding protein 1
XIAP	X-chromosome-linked inhibitor of apoptosis protein

List of included papers

(I) **Kanutte Huse**, Maren Bakkebø, Lise Forfang, Vera I. Hilden, Erlend B. Smeland, June H. Myklebust. Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: differential effects of BMP-6 and BMP-7. (submitted)

(II) Einar A. Sivertsen, **Kanutte Huse**, Marit E. Hystad, Christian Kersten, Erlend B. Smeland, June H. Myklebust. Inhibitory effects and target genes of bone morphogenetic protein 6 in Jurkat TAg cells. Eur J Immol 2007. 37:2937-48.

(III) **Kanutte Huse**, Maren Bakkebø, Morten P. Oksvold, Sébastien Wälchli, Vera I. Hilden, Lise Forfang, Knut Liestøl, Erlend B. Smeland, June H. Myklebust. Resistance to bone morphogenetic protein-induced growth inhibition in B-cell lymphoma. (submitted)

Introduction

1.1 The adaptive immune system

The immune system has evolved to protect the host against viruses, bacteria and parasites. An innate immune system is present in species of almost every level of evolution, showing its importance for survival [1]. The innate immune system relies on germline-encoded pattern receptors to recognize pathogens or damaged self components, such as the Toll-like receptors and nucleotide-binding domain leucine-rich repeat-containing receptors. The overwhelming variability of antigenic structures led to the need for a more flexible immune system, and adaptive immunity appeared in vertebrates around 500 million years ago. However, the innate immune system is still important as first level of protection and has a role in triggering antigen-specific responses by the adaptive immune system.

The cellular basis of the adaptive immune system is T cells, the effectors of the cellular immune response, and immunoglobulin (Ig) producing B cells, effectors of the humoral immune response [2;3]. These cells are activated through interplay with antigen presenting cells (APCs). During development of the B and T cells, the gene segments in their receptors are rearranged, generating a huge repertoire of specificities capable of recognizing any potential pathogen. This wide repertoire opens up for possibilities to create unwanted attacks against self-components, and brings the need for regulatory systems to avoid such attacks. The ability to differ between self and non-self is crucial for the immune system. Another important feature of the adaptive immune system is the generation of immunological memory. This enables the host to learn from encounters with various pathogens, leading to a more rapid and effective response to subsequent challenges with the same pathogen.

1.1.1 T cells

T cells recognize peptides in major histocompatibility complex (MHC)-peptide complexes [3]. All nucleated cells express MHC class I, which display peptides of all proteins, both normal (self) and microbial invaders (non-self) that are synthesized inside the cell. MHC class II are expressed on APCs, and they display peptides of microbes that are ingested by the APC. After MHC-peptide recognition, T cells mediate the immune response either by direct killing of an infected cell or by activating other immune cells.

1.1.1.1 Development of T cells

All cells of the immune system develop from hematopoietic stem cells in the bone marrow. T cells develop in the thymus from early progenitor cells that migrate from the bone marrow [2]. During the maturation steps in the thymus, the T-cell receptor (TCR) genes are rearranged, generating a T cell repertoire with wide diversity in MHC-peptide specificity [2;4]. Each TCR is made up of an α-chain and a β-chain (or γ- and δ-chain for a small subpopulation of T cells). The β-chain locus contains three regions, the variable (V), diversity (D) and joining (J) regions, each consisting of different gene segments. The α-chain only contains the V and J regions. During the rearrangement of the TCR, segments from each of the regions is randomly put together to create a V(D)J segment. Rearrangement continues until the V(D)J segment has been rearranged successfully, meaning that a protein product is made. If the thymocyte is not able to make a protein product of the TCR gene, the cell will die. Thymocytes with successful rearrangement will express a TCR on their surface and the specificity of the TCR will decide their further fate. When this is achieved, the rearrangement is stopped to secure that each thymocyte expresses TCRs of a single specificity.

Thymocytes with a newly made TCR will also express the co-receptors CD4 and CD8 and are now called double positive cells [5]. During a selection period, the TCR will bind to self MHC-self peptide complexes. Thymocytes binding strongly to self-antigen are eliminated to avoid autoimmunity, and thymocytes that do not bind self MHC-self peptide complexes are eliminated by lack of stimulation to ensure that the host has a repertoire of T cells that can bind its own MHC molecules. Only the thymocytes that bind weakly to self MHC-self peptide complexes are positively selected and stimulated to expand.

The further development of the thymocyte depends on which MHC class the thymocyte binds. There are two main categories of T cells, the $CD8^+$ cytotoxic T cells and the $CD4^+$ helper T cells (Th), with Th being the largest population. If the thymocyte binds MHC class I, it becomes a CD8 single positive cell and if it binds MHC class II it becomes a CD4 single positive cell. $CD8^+$ cytotoxic T cells recognize MHC class I and kill cells harbouring intracellular pathogens through contact-dependent mechanisms, inducing apoptosis in the target cell. $CD4^+$ Th cells recognize MHC class II and their function are to activate and direct other immune cells like the B cells. The CD4 and CD8 single positive T cells leave the thymus and will circulate between blood, lymph and secondary lymphoid organs until they encounter their cognate antigen. The antigen is presented in secondary lymphoid organs like a lymph node, in the form of MHC with peptide presented by an interacting cell.

1.1.1.2 $CD4^+$ T cell subsets

The naive $CD4^+$ T cells can further develop into several different effector subsets, depending on activation conditions. The different subtypes of Th cells are classified by their function and by the cytokines they produce (Fig. 1). Th1 cells are characterized by their production of INF-γ and IL-2 and they drive cell-mediated responses against intracellular pathogens, activating mononuclear phagocytes, NK cells and cytotoxic T cells [2;6]. Differentiation to Th1 cells are promoted by IL-12 and INF-γ. The other main type of Th cells, are the Th2 cells which produce IL-4, IL-5, IL-10 and IL-13. The cytokines produced by Th2 cells mainly activate B cells to enhance Ig production, and Th2 differentiation is induced by IL-2 and IL-4. Regulatory T cells (Tregs) are characterized by the expression of Foxp3 and are important suppressors of the immune system, thereby maintaining immune system homeostasis and tolerance to self-antigens [7]. They can either arise in the thymus (natural Tregs) or the Foxp3 expression can be induced by TGF-β in the periphery (induced Treg, iTreg). Some smaller Th subsets have also been discovered, for instance Th17 cells which participate in autoimmunity and produce IL-17, an inducer of many inflammatory cytokines [6]. The stability of the different CD4 Th subsets differs, and plasticity has been observed. For instance, there is a flexible balance between Treg and Th17 differentiation, and cells of both subsets can further differentiate to Th1 cells [8]. However, it has not been confirmed if the observed plasticity is a result of truly flexible genetic programs, or rather a display of heterogeneous populations.

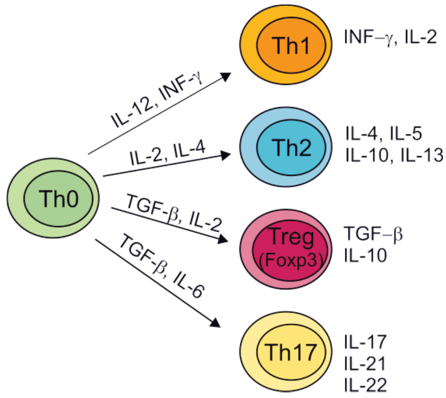

Figure 1: Different subsets of CD4$^+$ T helper cells

Specific cytokines direct the differentiation of progenitor T cells (Th0) into functionally distinct T cell subsets. Characteristic transcription factors and the main cytokines produced by each subset are also shown. (Modified from Bonilla and Oettgen, 2010 [2])

1.1.2 B cells

B cells are responsible for the humoral immune response, producing immunoglobulins (Igs) [3;9]. Igs will bind to pathogens, marking them for removal by cells or systems of the innate immune system. The B cell receptor complex (BCR) consists of an antigen-binding part, which is membrane bound Ig, and a signaling part which includes CD79α and β [10]. The Ig consists of a variable region, which interacts with antigen, and a constant region, which mediates the effector functions of the Ig.

1.1.2.1 Early B-cell development in the bone marrow

B cells develop in the bone marrow and the Ig is rearranged in much the same way as the TCR. Similar to the TCR gene segments, the variable regions of the Ig heavy (IgH) and light chains (IgL) also go through V(D)J rearrangement to create a wide repertoire of specificities [11]. The V(D)J recombination occurs in an ordered sequence in which the IgH is rearranged before the IgL. Upon successful rearrangement of IgH, it will constitute parts of the pre-BCR and be expressed on the cell surface. The expression of the pre-BCR on a pre-B cell will stop further rearranging of the heavy chain locus. The pre-B cell will be positively selected and rearrangement of the light chain follows. Then, successful rearrangement of the IgL results in the expression of the BCRs on the surface of the immature B cell and now the cell will be subjected to negative selection. Hence, B cells binding strongly to self proteins are removed as for the T cells to avoid autoimmunity. When the B cells exit the bone marrow, they express IgM and IgD on the cell surface. They are now called naive B cells, and circulate the blood and lymphatic tissue.

1.1.2.2 Antigen-dependent differentiation of B cells

The further development of naive B cells in secondary lymphoid organs is dependent on the type of foreign antigen, which cytokines that are available and if the B cell receives activation of co-receptors. Some antigens can activate B cells and induce differentiation into Ig–producing plasma cells without the help of T cells [12]. These antigens are called T cell-independent. Most antigens are T cell-dependent, meaning that T cells must participate in the activation of the B cell.

For T cell-dependent antigens, B cells require two signals to be activated [2]. The first signal is delivered by antigen binding to the BCR. Signaling pathways in the B cell will then be activated, preparing the B cell for the second signal which is delivered by activated Th cells. Th cells are activated by TCR binding to MHC class II molecules on APCs, and if the MHC molecule carries a peptide from an antigen corresponding to the antigen that activated the B cell, the T cell can provide help to the B cells through direct cell-cell contact via CD40L-CD40 binding. The B cell can then either become a short lived plasma cell secreting low-affinity antibodies, or it can establish a germinal center (GC) in a follicle (Fig. 2).

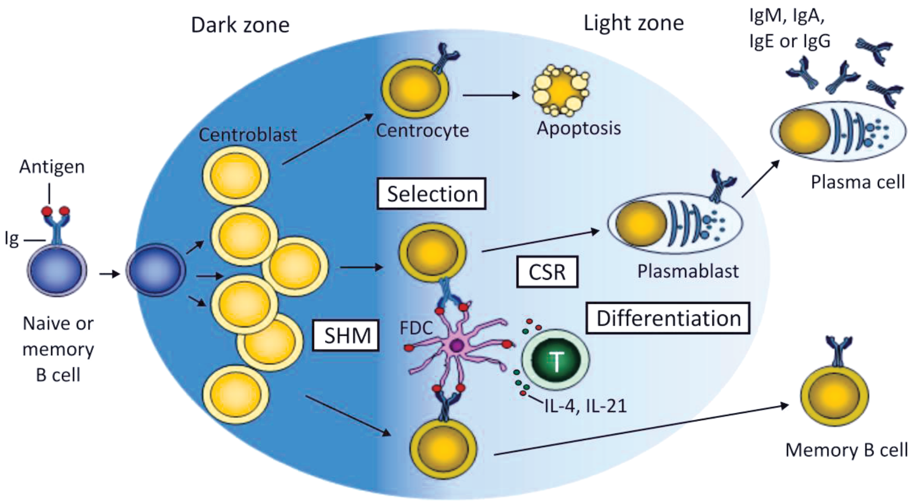

Figure 2. B-cell differentiation in the germinal center

Antigen-activated B cells differentiate to centroblasts and proliferate rapidly in the dark zone of the germinal center while the variable regions of the heavy and light chain are changed by somatic hypermutation (SHM). Centroblasts then differentiate to centrocytes and move to the light zone. Only centroblasts with improved affinity for the antigen are rescued from apoptosis by T cells and follicular dendritic cells (FDC). Some centrocytes undergo class switch recombination (CSR), directed by T cell-produced factors like interleukins, and after cycling between dark and light zones, antigen-selected centrocytes differentiate into memory B cells or plasmablasts. (Modified from Klein and Dalla Favera, 2008 [13]).

The main processes in a germinal center are affinity maturation of the Ig, changing of the Ig isotype and expansion of the high affinity B cells [13]. To change the isotype of the Ig, the B cell goes through a process similar to the V(D)J recombination, called class switch recombination (CSR). During CSR, the gene segments for IgM and IgD are cut and the variable region is ligated to a constant region of either IgG, IgA or IgE. The result is an Ig with the same specificity, but with a new constant region isotype. CSR is regulated by cytokines like IL-2, IL-4, IL-10 and TGF-β, and cellular interactions involving CD40 and CD40L [14]. For instance, IL-4 can induce *in vitro* production of IgG4 and IgE in CD40L-activated B cells [15], and IL-10 in combination with TGF-β can induce IgA production [16]. Recently, IL-21 has emerged as a potent inducer of Ig production when combined with CD40L [17;18], inducing secretion of IgG, IgA, IgE and IgM [19-21]. The enzyme activation-induced deaminase (AID) is required for CSR [22]. AID is a cytidine deaminase that converts cytosine to uracil, further resulting in double strand breaks [13]. AID is also involved in somatic hypermutation (SHM) [22], the process leading to affinity maturation. During SHM, point mutations and small deletions/insertions in the hypervariable domains of Ig heavy and light chains change the binding affinity for antigen [13]. Only the B cells with improved affinity binding will be positively selected. In contrast, B cells with Igs that after SHM bind antigen with less affinity, will lose in competition for binding to antigen present in immune complexes bound to follicular dendritic cells (FDCs), and will therefore not receive survival signals from the FDCs and will subsequently undergo apoptosis.

B cells surviving the GC reaction differentiate into either plasma cells or memory cells [23]. Plasma cells are terminally differentiated effector cells that secrete Ig and have lost their surface expression of BCR. Some plasma cells will migrate to the bone marrow and survive in specialized niches as long lived plasma cells, contributing to the humoral memory [12]. Memory B cells do not secrete Ig, but compared to naive cells, they have high affinity BCR and can quickly differentiate into plasma cells upon a recall response [23]. Pathogen re-exposure is the most effective way to maintain humoral memory, but BCR-independent polyclonal stimulation can also drive proliferation and differentiation of memory B cells.

1.1.2.3 Transcriptional regulation of B-cell development

The different stages of B-cell development are tightly regulated by transcription factors (Fig. 3). In the early developmental stages in the bone marrow, a range of transcription factors are involved, including PU.1, E2A, EBF and Pax5 which are important for lymphoid and B lineage commitment [24]. Pax5 controls the identity of B cells throughout B-cell development and is expressed until the plasma cell stage [25]. During the GC stage, Pax5 is required for upregulation of AID [26]. The transcriptional repressor BCL-6 is induced at high levels in GC B cells and is required for GC formation and induction of AID [27]. BCL-6 represses the expression of the DNA damage sensors p53, ATR and CHEK1, thereby allowing the GC B cells to tolerate the high level of DNA breaks introduced in their Ig genes by the processes of SHM and CSR. In addition, BCL-6 inhibits the cell cycle inhibitors p21 and p27, thus enabling centroblasts to undergo rapid proliferation. Another primary function of BCL-6 is to repress B-lymphocyte-induced maturation protein 1 (Blimp-1) – the master regulator of plasma cell differentiation [28]. Blimp-1 suppresses several genes involved in proliferation and GC functions, including Pax5 and BCL-6 [29].

Both these genes must be downregulated to allow terminal plasmacytic differentiation, and the mutual repression of Blimp-1 and BCL-6 forms a feedback loop enforcing irreversible differentiation [29;30]. By suppressing Pax5, Blimp-1 indirectly induces expression of X-box binding protein 1 (XBP-1) which is normally suppressed by Pax5 and is necessary to enhance the secretory capacity of plasma cells [31;32]. The transcription factor interferon regulatory factor 4 (IRF4), functioning upstream of XBP-1, is also required for plasma cell differentiation and an important role for IRF4 is to repress BCL-6 [33;34].

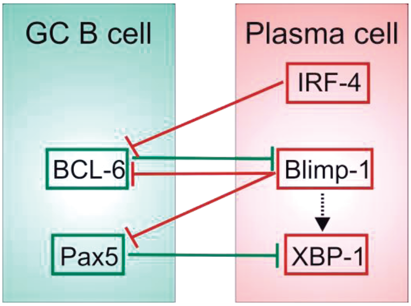

Figure 3: Regulation of transcription factors involved in plasma cell differentiation
The shown transcription factors are highly expressed in either GC B cells or plasma cells, and are important regulators of plasma cell differentiation. Their mutual repression ensures exclusive B cell and plasma cell gene expression programs.

1.1.2.4 Molecular markers identify distinct stages of B-cell maturation

Throughout B-cell development, the different maturation stages of B cells can be recognized by expression of stage specific surface molecules (Fig. 4). CD19 and CD20 are used as general B cell markers [35]. CD19 is a B cell co-receptor which is expressed from early bone marrow development and at all developmental stages except the terminally differentiated plasma cell stage. CD20 is also expressed on all mature B cells, but is downregulated in plasma cells. The isotype of the Ig indicates if the B cell has gone through a GC reaction with CSR or not. Naive B cells express IgD and IgM, but these molecules are lost during CSR in the GC, and post-GC cells express IgG, IgA or IgE. The TNF receptor protein CD27 is used to discriminate between naive and memory B cells [36]. Although a small population of IgG memory B cells are $CD27^-$ [37], most memory cells are $CD27^+$. The $CD27^+$ population contains both switched and non-switched memory B cells. Non-switched, also called IgM memory B cells, constitute about 20% of human peripheral B cells and are $IgM^+IgD^{-/+}CD27^+$ memory cells with mutated Ig genes [38]. The origin of these cells is still controversial. Furthermore, CD27 is involved in plasma cell differentiation and is gradually upregulated as GC B cells differentiate to plasma cells [39;40]. Plasma cells are also identified by the expression of CD38, which is induced in GC B cells and gradually upregulated when B cells differentiate to plasma cells [41]. CD138 is another plasma cell marker which is expressed in terminally differentiated plasma cells in the bone marrow and only weakly expressed in plasmablasts [42]. There are two B cell subsets in the GC. The rapidly dividing centroblasts and the resting centrocytes are two sequential stages where the Igs are undergoing SHM and CSR, respectively [43;44]. These B cell subsets are both $CD38^+IgD^-$ and can be distinguished by the expression of CD77 (see Fig. 4). However, later studies have indicated that CD77 is not a good discriminator between the two populations as the gene expression profiles of $CD77^-$ and $CD77^+$ cells were found to be very similar and their functional characteristics do not reflect what is known for centrocytes and centroblasts [45].

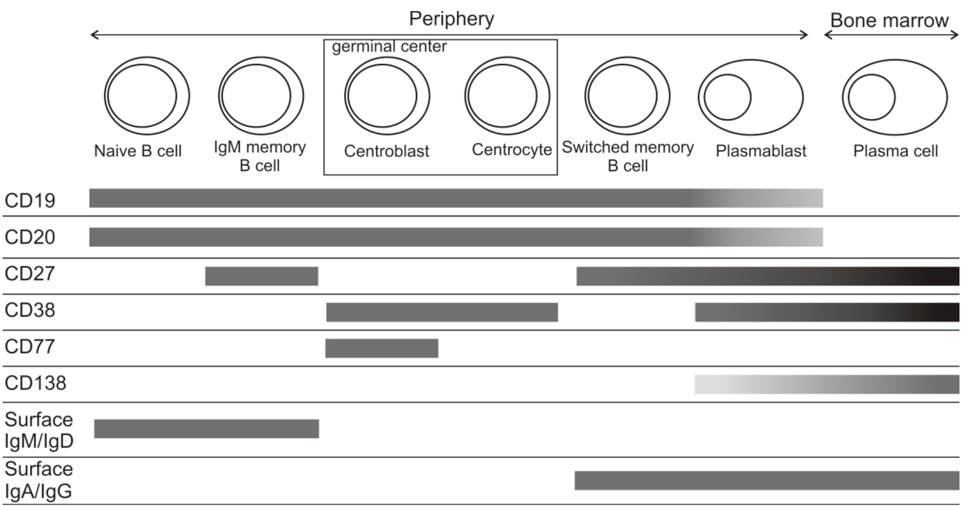

Figure 4: Surface markers distinguish the various B-cell maturation stages

Expression of surface markers can distinguish different maturation stages of B cells in the periphery and the bone marrow. Darkness of line indicates weak (light grey) or strong (dark grey) expression of molecule.

1.2 B-cell lymphoma

Malignant lymphomas are cancer that arise from lymphocytes and present as tumors in lymphatic tissue. Lymphomas comprise about 3% of all new cancer cases per year [46]. About 95% of the lymphomas are of B cell origin, the rest are T-cell and natural killer cell malignancies [47]. There are two main categories of B-cell lymphoma, Hodgkin's lymphoma and Non-Hodgkin lymphoma (NHL). NHL are further divided into several subtypes (see section 1.2.2) of which diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are most common and account for about 60% of cases.

1.2.1 Mechanisms of lymphomagenesis

Like other cancers, B-cell lymphomas are caused by activation of proto-oncogenes or deletion of tumor suppressor genes [48]. This can disrupt the normal B cell homeostasis in three ways: inducing cell cycle progression, preventing cell death and blocking differentiation (Fig. 5). A hallmark of most B-cell lymphomas is chromosomal translocations where an oncogene comes under the control of Ig enhancers [47]. The consequence of such a translocation is that the oncogene is deregulated and constitutively expressed. During the development of the B cell, the Ig gene is subjected to DNA double strand breaks during V(D)J rearrangement, CSR and SHM, as described in sections 1.1.2.1 and 1.1.2.2. These processes secure the diversity of the immune responses, but also make the cells susceptible to genetic alterations like translocations. Both CSR and SHM occur in the GC, which might explain why most B-cell NHL types resemble GC or post-GC B cells.

Positioning of the breakpoint in an Ig translocation indicates at what stage of B-cell development the mistake leading to the translocation has occurred [49]. Translocations with breakpoints close to the J-region of the Ig heavy chain (IgH), such as the t(14:18) translocation involving BCL-2 and IgH, is thought to happen as a mistake during V(D)J recombination. The t(10:14) translocation involving BCL-6 and IgH is thought to happen as a mistake of CSR since the breakpoint is located in the switch regions were DNA breaks are introduced during CSR. Furthermore, some c-myc translocations are thought to happen as a by-product of SHM since the breakpoints are located within rearranged VJ-genes or in the J intron sequence [50].

BCL-2 is an anti-apoptotic factor that is normally not expressed by centroblasts [51]. It is thought that ectopic expression of BCL-2 as a result of chromosomal translocations overrides the characteristic pro-apoptotic program of the centroblasts, thereby promoting lymphomagenesis [13]. However, the BCL-2 translocations can also be found in B cells of healthy humans, although at a low frequency ($1 \times 10^{-5} – 1 \times 10^{-6}$) [52;53]. These findings imply that a chromosomal translocation is not sufficient to induce lymphomagenesis, but that the translocation is part of a multistep transformation process.

In addition to deregulation by chromosomal translocation, BCL-6 can be deregulated as a result of SHM. BCL-6 is one of the non-Ig gene targets which are affected by SHM, and hypermutated BCL-6 genes are found in both healthy individuals and in lymphomas [54;55]. BCL-6 is normally expressed in GC B cells, but must be downregulated to end the GC reaction [27] (see section 1.1.2.3). Constitutive activation of BCL-6 prevents differentiation to a post-GC B cell, and keeps the cell in a highly proliferative state [13]. BCL-6 also has an oncogenic role as regulator of DNA damage and apoptotic responses in GC B cells [27]. As mentioned (section 1.1.2.3), BCL-6 expression makes the GC B cells tolerable to the genomic stress of rapid proliferation and DNA remodeling without activating DNA damage responses via suppression of p53 and ATR. This state of tolerance also makes them susceptible to genetic alterations leading to lymphomagenesis.

Another mechanism of genetic instability is aberrant somatic hypermutation (ASHM). In DLBCL, ASHM targeting well-known proto-oncogenes like PIM1, c-myc and Pax5 is identified in more than 50% of the cases [56]. In contrast to SHM of BCL-6, mutations of these genes do not occur in normal GC B cells, suggesting a malfunction of SHM.

B-cell lymphomas are not autonomous, but rely on the same key factors for survival as normal B cells [47]. Most B-cell lymphomas express BCRs, and as translocations almost exclusively are found on the non-productively rearranged Ig loci, this indicates that BCR-derived survival signals are crucial for the malignant B cells. Also, in lymphomas with ongoing SHM, the BCR expression is retained. Furthermore, CSR and SHM are dependent on AID, suggesting a role for AID in lymphomagenesis [57]. AID is constitutively expressed in most GC-derived B-cell lymphomas, and AID deficient mice do not develop BCL-6-dependent, GC-derived lymphomas, but can develop c-myc-driven, pre-GC lymphomas [58]. However, AID mutations are not found in B-NHL, indicating that malfunctions in CSR and SHM are not directly caused by AID but possibly by alterations in the mechanisms regulating AID expression [13].

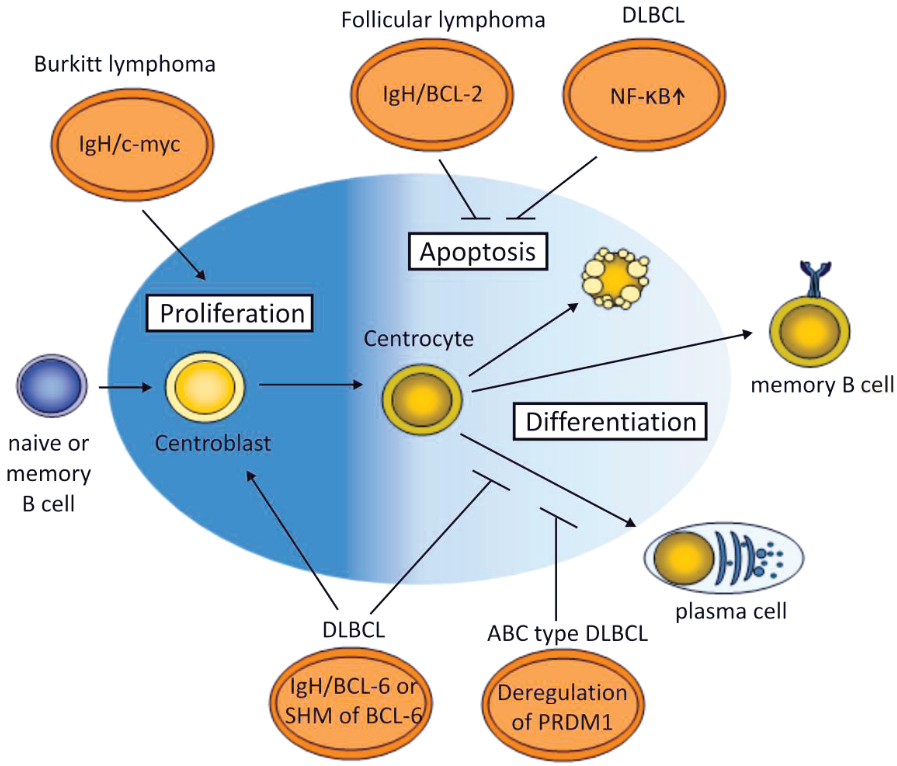

Figure 5. Molecular mechanisms of lymphomagenesis

The figure shows examples of proto-oncogenes and a tumor suppressor which can cause lymphoma by inducing proliferation, inhibiting apoptosis or blocking differentiation. Deregulation of c-myc as a result of chromosomal translocations may give the B cell a proliferative advantage. The same effect can occur after deregulation of BCL-6 as a result of either chromosomal translocations or SHM of the regulatory region of BCL-6. Deregulation of BCL-6 or PRDM1 can prevent differentiation, thereby keeping the cell in the GC stage. Constitutive expression of BCL-2 caused by chromosomal translocations, or constitutive NF-κB activation result in inhibition of apoptosis. The figure also indicates in which lymphoma histologies the alterations are found and the percentage of cases known to carry the alteration [47]. (Modified from Klein and Dalla Favera, 2008 [13]).

1.2.2 Classification of lymphoma subtypes

The different lymphoma subtypes differ in aggressiveness and curability. Thus, to optimize treatment and improve clinical outcome, it is important to find the correct diagnosis in each case. The universally accepted World Health Organization classification of lymphomas relies on a combination of clinical, morphological, immunophenotypic, genetic and other biological features to define the different disease entities [59]. Most B-cell malignancies seem to be "frozen" at a particular stage of normal B-cell development which reflects their "cell of origin" [60]. Analysis of the Ig genes can be used to identify the cell of origin, as the presence of Ig mutations is usually taken as evidence that the cell has been through the GC reaction [61]. Recently, the use of genomic-scale gene-expression profiling has clarified the relationship between B-cell malignancies and their normal counterparts. Different stages of B-cell differentiation, like GC B cells, can be identified by specific expression signatures [62;63].

1.2.2.1 Diffuse large B-cell lymphoma

DLBCL is the largest category of NHL, accounting for approximately 40% of all cases [62]. The disease is both clinically and molecularly heterogeneous. Several mechanisms of pathogenesis are reported, like chromosomal translocations including BCL-6, BCL-2 or c-myc, and inactivation of PRDM1, leading to blocked differentiation [13]. Furthermore, ASHM of proto-oncogenes is found in about half of all DLBCL cases [47]. Gene expression profiling distinguished at least two subtypes of DLBCL which also defined different prognostic categories [62;64].The most favourable group of these two categories expressed genes that were hallmarks of normal GC B cells and were called the germinal center B-cell type (GCB). The less favourable activated B-cell subtype (ABC) expressed many of the genes that are induced during *in vitro* activation of peripheral blood B cells. A third subtype of DLBCL, primary mediastinal B-cell lymphoma, was recognized later. It shares clinical and genetic features with Hodgkin's lymphoma, typically arises in younger patients and has a favourable outcome [65;66]. An important difference between the subtypes is the constitutive active NF-κB pathway in ABC and PMBL but not in GCB [67].

1.2.2.2 Follicular lymphoma

FL is the second most frequent NHL, accounting for about 20% of all lymphoma cases [68]. The hallmark of FL is the BCL-2 translocation, resulting in protection from apoptosis. FL is derived from GC B cells, maintaining the gene expression of these cells, and the malignant B cells of FL have ongoing SHM. Some patients have an aggressive disease and die within a year, but FL typically follows an indolent course with a median survival of 8 – 10 years, although with frequent relapses. Ultimately, either resistance to chemotherapy or transformation to the more aggressive DLBCL results in the patient dying from the disease.

Gene expression profiling has revealed that prognosis of FL can be predicted at time of prognosis by two gene signatures termed immune-response 1 and immune-response 2 [69]. The genes included in the signatures are preferentially expressed by the non-malignant, $CD19^-$ cells, indicating a significant role of the microenvironment in tumor development and progression. Immune-response 1, which is associated with good prognosis, is composed of genes mostly expressed by T cells. The immune-response 2 signature reflects immune infiltrate dominated by macrophages and dendritic cells and is associated with poor outcome. Many FL express both signatures, and their relative ratio is what predicts survival [67;70].

1.2.2.3 Burkitt lymphoma

Burkitt lymphoma (BL) is an aggressive B-cell lymphoma, primarily affecting children and young adults [71]. There are three variants of BL which are histological indistinguishable: endemic (eBL), sporadic (sBL) and immunodeficiency-associated BL. eBL generally affects children in tropical Africa where co-infection of malaria parasites seems to be an important co-factor. sBL occurs worldwide, but at a much lower prevalence. In HIV-positive adults and other patients with immunodeficiency, the coincidence of BL is high.

All subtypes of BL are characterized by translocations involving the c-myc oncogene and Ig genes, but the breakpoints in the Ig genes vary between the subtypes [72]. Deregulation of c-myc leads to cell growth and proliferation in addition to reduced threshold for induction of apoptosis, and the latter must be repressed for lymphomas to develop. In more than 90% of eBL and 25 - 40% of immunodeficiency-associated BL, Epstein-Barr virus (EBV) is involved, probably repressing pathways leading to apoptosis. In sBL, where EBV involvement is less common, a second oncogenic lesion must occur to repress apoptosis. The low probability for this to happen might account for the general rarity of sBL [71].

1.3 Bone morphogenetic proteins – critical regulators of homeostasis

Bone morphogenetic proteins (BMPs) are part of the TGF-β superfamily which also includes TGF-β, activin/inhibin and growth and differentiation factors (GDFs). More than 20 BMP-related proteins have been identified, and these can be further subdivided into groups based on amino-acid structure and functions [73]. BMPs were originally identified by their ability to induce formation of bone and cartilage [74;75]. Later, it has been found that BMPs also play roles in a number of non-osteogenic developmental processes, including embryonic development and tissue homeostasis in adults. BMPs are shown to regulate proliferation, differentiation, migration and apoptosis in various cell types including neural cells and epithelial cells [76]. Hematopoietic cells can also be influenced by BMPs. In this respect, BMP-4 has been shown to act as a survival factor for hematopoietic stem cells from both adult and neonatal sources [77], and BMP-6 was shown to inhibit the growth of human peripheral blood B cells [78].

Studies of knockout mice confirm the important role of BMPs during development. BMP-2 and BMP-4 deficient mice are nonviable due to defects in cardiac development and mesoderm differentiation, respectively [79]. BMP-7 deficient mice display defects in the development of kidney, skeleton and eye, and die shortly after birth [80]. Knock-out of BMP receptors or Smads also leads to lethality or serious defects [79]. BMP-4 has a crucial role in hematopoietic commitment during embryonic development in several species [81]. For instance, BMP-4 promotes hematopoietic differentiation of human embryonic stem cells [82].

1.3.1 BMP signaling and target genes

BMPs and the other TGF-β superfamily ligands induce activation of signaling pathways that have many common signaling molecules. The signaling pathway of BMP involves the BMP ligand, two types of serine/threonin receptors and the Smad-proteins (Fig. 6). In humans, there are six receptors known to bind BMP, the type I receptors activin receptor-like kinase (Alk) 2, Alk3, Alk6 and the type II receptors BMP receptor II (BMPRII), activin receptor II (ActRII) A and ActRIIB [76]. The type II receptors are constitutively active, whereas the type I receptors require ligand binding, ligand-receptor oligomerization and transphosphorylation via type II receptor to be activated. The activated type I receptor will phosphorylate the receptor-regulated Smads (R-Smads): Smad1, Smad5 and Smad8. Furthermore, R-Smads will make a trimeric complex with Smad4 [83] and translocate to the nucleus to bind DNA and regulate transcription of target genes.

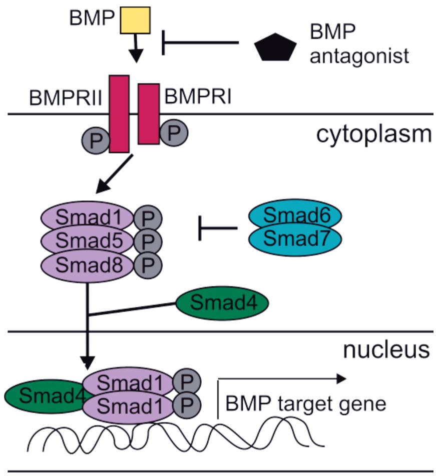

Figure 6: The Smad-dependent BMP signaling pathway

The BMP ligand binds a BMP receptor complex which leads to activation of the Smad signaling pathway. The activated receptor complex phosphorylates R-Smads (Smad1/5/8) which form a complex with Smad4 and translocate to the nucleus where they regulate gene transcription. BMP antagonists and inhibitory Smads (Smad6/7) can inhibit BMP signaling. (Modified from Wordinger et al. 2002 [84])

Several mechanisms exist for negative regulation of the BMP pathway. Among these are the inhibitory Smads, Smad6 and Smad7. Inhibitory Smads can interact with activated BMP type I receptors, preventing the R-Smads to bind the receptor [85]. Smad6 can also compete with Smad4 for heteromeric complex formation with activated R-Smads, and Smad7 has been shown to interact with ubiquitin ligases, leading to proteosomal and lysosomal degradation of Smad proteins [85]. Extracellular BMP antagonists like noggin and chordin are shown to bind BMP ligands with different affinities, thereby preventing binding to receptors and suppressing BMP signaling [86]. Both inhibitory Smads and BMP antagonists can be induced by BMPs, showing their role in the natural negative feedback mechanism of BMPs [87-89].

Inhibitor of differentiation (Id) proteins are considered to be some of the most important target genes of BMP signaling, mediating many of the biological responses induced by BMPs in various cells [90]. They are negative regulators of basic helix-loop-helix transcription factors, and generally induce proliferation but inhibit differentiation. Runx2 is also a BMP target gene, and Runx is a family of transcription factors that interacts with R-Smads and regulates various biological events, including hematopoiesis and bone formation [91]. Examples of other BMP target genes were seen during BMP-2-induced osteoblast differentiation. This led to upregulation of several genes involved in signal transduction, including TIEG and inhibitory Smads, in addition to the transcription factors Hey1 and Tcf7 which are involved in Notch and Wnt signaling [92].

In addition to signaling through the Smad pathway, BMPs can also induce Smad-independent signaling. BMP receptors can associate with TAK1 (TGF-β activated kinase 1), TAB1 (TAK1 binding protein 1) and XIAP (X-chromosome-linked inhibitor of apoptosis protein), thereby activating MAPK pathways [93]. Studies have shown that the oligomerization of BMP receptor complexes determines if Smad or non-Smad pathways are activated [94;95]. When BMP-2 binds to preformed BMP receptor complexes, Smad signaling is induced, whereas binding to BMP-2-induced receptor complexes results in p38 MAPK signaling. Through activation of p38 MAPK and JNK, BMPs can lead to apoptosis in several cell types, and this response can be blocked by Smad6 and Smad7 [96]. BMPs are necessary during osteoblast differentiation, and in this setting BMP stimulation has been shown to activate ERK signaling in addition to p38 and JNK [96]. MAPK is also involved in BMP signaling through cross-talk with Smad proteins [97]. ERK1/2 can phosphorylate the linker region in Smad1/5, thereby blocking nuclear translocation or activate Smad ubiquitin regulatory factors 1 (Smurf1), leading to ubiquitination and degradation of Smad1/5. Cross talk between the BMP and Wnt pathways is well studied, and the two pathways are interwined throughout life, interacting at several levels [97]. For instance, BMP and Wnt regulate each other’s ligand production, which is critical for establishing extracellular gradients during embryonic development. Many other pathways can cross-talk with the BMP signaling pathway, contributing to the cell type and context dependence seen in responses to BMP [97].

1.3.2 The role of BMP signaling in cancer

Biological responses to BMP signaling are highly cell type specific, and this also holds true for malignant cells. Whereas some studies show that BMPs can promote tumorigenesis and metastasis, others show that BMPs can have negative effects on cancer [98;99]. Studies performed in multiple myeloma, cancer that originates from plasma cells, report that BMP-2, -4, -6 and -7 inhibit proliferation and induce cell death in myeloma cells [100-102]. In contrast, the role of BMPs in B-cell lymphoma is largely unknown.

As BMPs are essential regulators of many important cellular processes, appropriate regulation of the BMP signaling pathway is important at all levels and alterations in the pathway can result in cancer [103]. Genetic studies in familial cancer syndromes give convincing indications that the BMP signaling pathway is involved in carcinogenesis. Germ-line mutations of Smad4 and Alk3 have each been identified in approximately 20% of cases of familial juvenile polyposis [104;105]. Additionally, mutation in Alk3 has been reported in a patient with symptoms of Cowden and Bannayan-Riley-Ruvalcaba syndrome [106]. Alterations in the BMP signaling pathway are also found in sporadic cancers, and Figure 7 shows an overview of alterations found in human cancers, including alterations in the TGF-β signaling pathway as this pathway has been more extensively studied than the BMP pathway.

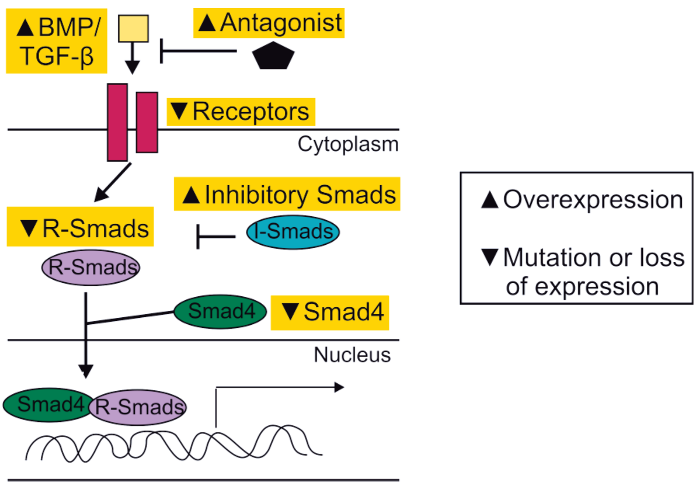

Figure 7. Alterations of the BMP and TGF-β signaling pathways found in human cancer. Reviewed in Levy and Hill, 2006 [103].

2 Aims of the present study

The well known role of TGF-β as an important regulator of immune responses is a good rationale to perform studies of related molecules, like BMPs. Studying the effects of BMPs on proliferation and differentiation of B and T cells will shed light on the complex regulatory pathways utilized to ensure appropriate generation of optimal immune responses. If BMPs can affect proliferation and cell survival of normal lymphocytes, it will also be interesting to investigate a possible role for BMPs in the malignant counterparts.

The aim of this study has been to elucidate the role of BMPs in normal and malignant lymphocytes. To do this, we investigated the functional effects of BMPs in B and T cells, including effects on proliferation, apoptosis and differentiation. Further, we wanted to identify BMP target genes and BMP signaling in B and T cells. In more detail, the study was designed to:

1) Investigate the immunoregulatory role of BMPs in mature, human B cells by observing effects on Ig production, differentiation and cell growth, and explore the molecular mechanisms of these effects.

2) Identify BMP-6 target genes in human T cells using microarray technology, and to study the functional effects of BMP-6 in these cells.

3) Study if B-cell lymphomas can escape the negative influence of BMPs and if so, explore the molecular mechanisms for BMP resistance.

3 Summary of included papers

Paper I: Bone morphogenetic proteins inhibit CD40L/IL-21-induced Ig production in human B cells: differential effects of BMP-6 and BMP-7

In this paper we studied the effects of various BMPs in naive and memory B cells from peripheral blood of healthy human donors. We used immunomagnetic beads and FACS to purify $CD19^{+}CD27^{-}$ naive and $CD19^{+}CD27^{+}$ memory B cells, and investigated BMP induced effects on *in vitro* proliferation, cell death and Ig production. To separate direct inhibition of plasma cell differentiation from indirect effects via suppression of proliferation and induction of apoptosis, CFSE tracking of cell division was combined with immunophenotyping of cultured cells to identify $CD27^{+}CD38^{+}$ plasma cells. We observed that BMP-2, -4, -6 and -7 specifically inhibited CD40L/IL-21-induced production of IgM, IgG and IgA. BMP-6 was the most potent inhibitor, reducing the Ig production by 70% in memory B cells and more than 50% in naive B cells. By investigating the mechanisms for reduced Ig production, we found a striking difference between the structurally similar BMP-6 and BMP-7. BMP-6 directly inhibited differentiation to $CD27^{+}CD38^{+}$ plasmablasts, whereas BMP-7 only had minor effects on differentiation. Instead, BMP-7 mainly affected Ig production indirectly by inducing apoptosis. Furthermore, we explored BMP-6-induced signaling and gene regulation in more detail in memory B cells. BMP-6 upregulated Id1, Id2 and Id3 gene expression in CD40L/IL-21-stimulated cells. In contrast, BMP-6 potently inhibited CD40L/IL-21-induced upregulation of the transcription factor XBP-1, necessary for the late stages of plasmacytic differentiation. Expression of transcription factors regulating earlier stages (IRF4, PRDM1) was not affected, indicating that BMP-6 only modulates late events of plasma cell development. In conclusion, these results show that BMPs potently suppress Ig production in mature human B cells either by direct inhibition of differentiation (BMP-6) or indirectly via induction of apoptosis (BMP-7).

Paper II: Inhibitory effects and target genes of bone morphogenetic protein 6 in Jurkat TAg cells

In the present study we explored the role of BMP-6 in human T cells. We used $CD4^+$ T cells purified from peripheral blood of healthy donors and the T cell line Jurkat TAg. In Jurkat TAg cells, genome-wide expression profiling of cells treated with BMP-6 compared to medium showed that Id1-3 were target genes of BMP-6 together with Noggin and Smad6. Furthermore, several genes involved in transcriptional regulation were also identified as BMP-6 target genes, including NFKBIA, HEY1, DLX2, KLF10 and early growth response 1. Western blotting experiments confirmed that Id1 and Id3 proteins were upregulated by BMP-6. We further investigated BMP-6 induced signaling in Jurkat TAg cells, and found that BMP-6 activated canonical as well as non-canonical pathways as measured by rapid phosphorylation of Smad1/5/8 (canonical), and p38 and ERK1/2 (non-canonical). The role of Id1 as an important downstream mediator in T cells was further strengthened as BMP-6 exerted an anti-proliferative effect in Jurkat TAg cells that was counteracted by Id1 siRNA (measured by 3H-thymidine incorporation). In $CD4^+$ T cells, we also found a potent upregulation of Id1, Id2 and Id3 mRNA after BMP-6-treatment as measured by real-time RT PCR. However, in these cells no significant changes were observed in Id protein expression, possibly due to BMP effects only in a subset of T cells. Importantly, BMP receptor expression analysis as measured by FACS analysis supported this hypothesis, as Jurkat TAg cells, but only a subset of $CD4^+$ T cells, were found to express the BMP receptors Alk2 and Alk3 (type I), in addition to BMPRII (type II). Altogether, the data indicate a role for BMP-6 in human T lineage cells.

Paper III: Resistance to bone morphogenetic protein-induced growth inhibition in B-cell lymphoma

Initially, expression of BMP mRNA was observed in normal germinal center B cells, lymphoma cell lines and in malignant B cells purified from lymphoma patient samples, suggesting a physiological role of BMPs in normal as well as malignant B cells. Especially BMP-7 was highly expressed. Further, the functional influence of BMP-2, -4, -6 and -7 in human B-cell lymphomas was investigated by measuring DNA synthesis and cell death. Whereas BMPs strongly inhibited cell growth in some lymphoma cell lines with up to 90% inhibition of DNA synthesis, others were completely resistant to BMP-induced growth inhibition. By using Western blotting we measured BMP-induced phosphorylation of Smad1/5/8, and found that some primary tumor samples from lymphoma patients also were resistant to BMPs, as determined by lack of Smad1/5/8 activation. Furthermore, BMP-7 had no or limited effect in any of the lymphoma cells tested, which was interesting considering the high expression of BMP-7 mRNA in malignant B cells. Sensitive and resistant cell lines were further compared to find differences that could explain resistance to BMPs. Downregulation of BMP receptors is seen in many cancers, but we found similar receptor levels in resistant compared to sensitive lymphoma cells as determined by FACS analysis. We found a positive correlation between activation of Smad1/5/8 and inhibition of DNA synthesis, suggesting that the mechanism of resistance generally is upstream of Smad1/5/8 activation. Expression of R-Smads and inhibitory Smads was determined by Western blotting and real-time RT-PCR. Of the three resistant cell lines, we found that one expressed high levels of inhibitory Smad7 mRNA and one had low levels of Smad1 and Smad5. Analysis of gene expression data confirmed high expression of Smad7 in lymphoma patient samples. These findings suggest that downregulation of R-Smads and upregulation of inhibitory Smads are possible mechanisms for resistance.

4 Discussion

4.1 Metodological considerations

4.1.1 Cell systems

The cell systems used in paper I-III have been primary cells from healthy donors, tumor samples from lymphoma patients and cell lines. These cell systems have different strengths and weaknesses as model systems for normal and malignant lymphocytes.

Cell lines have the advantage of providing almost unlimited amounts of cells. They are also easy to culture and manipulations like transfections are often more feasible in these cells. Some lymphoma cell lines are very good models of their lymphoma subtypes. Gene expression studies of DLBCL including both primary tumors and lymphoma cell lines, identified cell lines which could represent the different subgroups on the basis of having similar gene expression profiles [62;107;108]. We have used several cell lines from these studies, including Sudhl-4, Sudhl-6, OCI-Ly3, OCI-Ly7, OCI-Ly10. Importantly, these cell lines have been used by others to identify crucial pathogenetic mechanisms in DLBCL [108;109]. In contrast, some cell lines have, after being cultured *in vitro* for several years, lost many of their original "*in vivo* characteristics" and the physiological relevance is therefore questionable. The cell lines may have acquired additional mutations, thus changing important pathways controlling proliferation and apoptosis. An example of this is cell lines of the FL subtype. *In vivo*, FL is dependent on interaction with stromal cells to grow [68]. In *in vitro* cultures, these cell lines grow independently of stromal cells which shows that they have changed compared to primary FL cells.

Considering the physiological relevance of *in vitro* studies, using primary cells from human donors or patients is a better alternative. However, primary cells survive *in vitro* only for limited time periods. They need to be activated by antigen receptor cross linking, cytokines and/or co-receptor stimulations in order to survive and proliferate, thus making experimental manipulations less feasible and the amount of cells is limited. Especially cells from tumor material were fragile after thawing and we were not able to do functional studies in these cells. There are also ethical and juridical regulations to consider when working with cells from patients and donors. In this respect, samples or biopsies from healthy donors or lymphoma patients were collected with informed consent in accordance with the Declaration of Helsinki and approved by the regional Committee for Medical Research Ethics, Region Eastern Norway.

4.1.2 Control of quality and specificity of recombinant human BMPs

Initially, we experienced that there were batch to batch variations of recombinant human (rh) BMP-6. For instance, a few batches did not show any biological activity at all. To overcome this problem, each new batch of rhBMP was tested in parallel with the old batch in Sudhl-6 cells using a 3H-thymidine incorporation assay. This was done to ensure that the quality of BMPs were as expected, prior to using them in experiments. Suboptimal batches of BMPs were not used for experiments.

The specificity of the BMPs was tested by using soluble receptors (paper II), the BMP antagonist Noggin (paper I and II), the small molecule inhibitor Dorsomorphin (paper III) or a neutralizing anti-BMP-6 antibody (paper I). Both Smad signaling and the inhibitory functional effects of BMPs were counteracted by the various agents used, showing that the effects studied were BMP specific effects. Of notice, the effects of BMP-6 in primary B and T cells were not counteracted by Noggin, but the specificity in B cells were shown by using a neutralizing anti-BMP-6 antibody (paper I).

4.1.3 Transfection of lymphocytes

Primary cells are generally difficult to transfect. In addition, the survival after transfection and transfection efficacies in the B-cell lymphoma cell lines used for this study varied, limiting our possibilities for knock-down of genes or overexpression studies. In Jurkat TAg cells, we were able to knock down BMP-6-induced Id1 expression by introducing Id1 siRNA by electroporation (paper II). In paper I, we hypothesized a possible role of Id1 as mediator of BMP effects in primary B cells. To strengthen this hypothesis, we tried to knock down Id1 in normal human B cells. For this purpose, we used an electroporator machine from Amaxa (Nucleofector), as the producer reported 40% transfection efficacy in unstimulated B cells. However, we only experienced an efficacy of less than 10% when transfecting with GFP plasmids. To overcome the problem with low transfection efficacy in primary B cells, we tested if pre-stimulation with CD40L/IL-21 would make the cells more easily transfectable. In fact, pre-stimulation for at least 16 hours before transfecting the cells increased the transfection efficacy more than 4-fold, compared to cells that had only been pre-stimulated for 3 h (Fig. 8). However, despite almost 50% transfection efficacy, we were not able to get reliable knock-down of Id1 mRNA although several different Id1 siRNA were tested, including the Id1 siRNA we successfully used for transfecting Jurkat TAg cells (paper II). Thus, we concluded that retroviral or possibly lentiviral transduction had to be used in order to genetically manipulate primary B cells, a method not yet established in our lab.

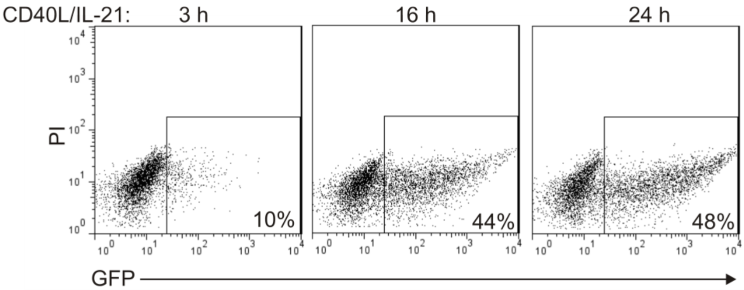

Figure 8. Pre-stimulation with CD40L/IL-21 increased the transfection efficacy in primary B cells.
To optimize transfection efficacy and survival, primary $CD19^+$ B cells from peripheral blood were stimulated with CD40L and IL-21 for various periods of time before transfection with maxGFP, using the Human B Cell Nucleofector Kit (Amaxa). Figure shows expression of GFP one day after transfection. Survival was measured with PI, and we observed 41, 54 and 51% live cells for cells pre-stimulated for 3, 16 and 24 hours, respectively.

In the B-cell lymphoma cell line ROS-50 which was one of the BMP resistant cell lines, we found high expression of Smad7 and hypothesized that upregulation of Smad7 was the resistance mechanism in this cell line (paper III). To confirm whether upregulation of Smad7 was the reason for BMP resistance, we used Smad7 siRNA to knock down Smad7. ROS-50 was one of the cell lines with high transfection efficacy after nucleofection (>70% GFP^+ cells 1 day after transfection). We were able to reduce Smad7 mRNA expression using 3 different Smad7 siRNAs, but this had no effect on Smad7 protein expression levels (Fig. 9). This finding suggested that Smad7 protein in ROS-50 was very stable and could not be downregulated by transient transfection. In line with this, Grönroos et al. have shown that acetylation of the Smad7 protein stabilizes it and protects it from ubiquitination and degradation [110]. Thus, the establishment of stable $Smad7^{-/-}$ ROS-50 clones, or to use retroviral transduction with Smad7 shRNA is needed to explore the role of Smad7 in B-cell lymphoma.

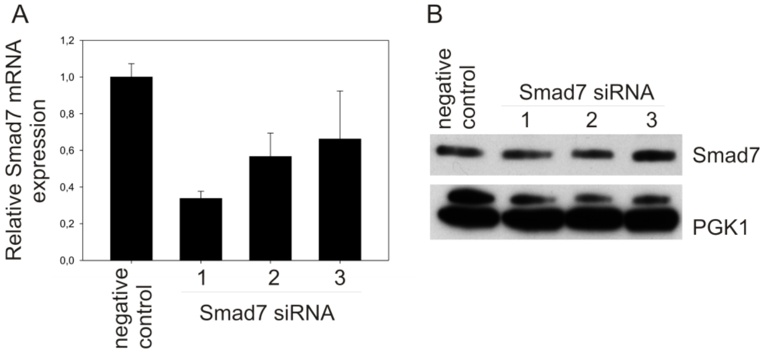

Figure 9. Smad7 siRNAs reduced Smad7 mRNA expression, but not protein expression. ROS-50 cells were transiently transfected with 2 µM Smad7 siRNA, using amaxa-technology (siRNA 1 (GCTCAATTCGGACAACAAG): purchased from ambion, siRNA 2 (GTGTTCAGGTGGCCGGATCT) and siRNA 3 (GGACGCTGTTGGTACACAA): constructed in-house). One day after transfection, Smad7 mRNA was reduced (A) whereas Smad7 protein expression was not affected (B).

4.2 Normal and malignant B cells express BMP mRNA

If studying the BMPs' effects in normal and malignant lymphocytes should be of physiological relevance, BMPs must be available for B and T cells in their microenvironment. In paper III, we investigated BMP mRNA expression in normal and malignant germinal center cells and found that both normal and malignant B cells expressed BMP-7, whereas the expression of BMP-4 and BMP-6 was limited. Infiltrating T cells sorted from lymphoma samples expressed low levels of BMP-6 and BMP-7, whereas BMP-2 expression was not detected in any of the cell types tested. In line with our findings, others have found expression of BMP-6 and BMP-7, but not BMP-2 and BMP-4, in the BL cell line Ramos [111;112] and BMP-6 was absent in DLBCL cell lines and the two BL cell lines Bjab and Raji [112]. BMP-4 expression has previously been found in the T cell lines Molt4, SupT1 and Jurkat [111]. We also observed BMP-6 mRNA expression in naive and memory B cells from peripheral blood (unpublished results), and previously we have shown expression of BMP-6 in $CD19^+$ cells from peripheral blood [78]. The expression of BMP-6 was increased upon treatment with anti-IgM or serum [78]. Interestingly, Detmer et al.

found low levels of BMP-7 mRNA expression in B and T cells from peripheral blood [111], and peripheral T cells, but not B cells, have been shown to express BMP-4 [113]. BMP expression has also been observed in normal and malignant plasma cells [114;115]. Taken together, this indicates that BMPs exist in lymphoid tissue and that the observed effects of BMPs on lymphocytes are of physiological relevance.

4.3 Functional influence of BMPs on normal lymphocytes

4.3.1 BMPs inhibit differentiation of B and T cells

We found that BMPs had inhibitory effects on normal human B- and T-cell differentiation. We used the combination of IL-21 and CD40L to induce differentiation of naive and memory B cells to Ig-secreting plasmablasts, and found that BMP-2, -4, -6 and -7 suppressed the production of IgM, IgA and IgG in both B cell subsets (paper I). By combining CFSE tracking of cell division with immunophenotyping to identify $CD27^+CD38^+$ plasmablasts, we could separate direct inhibition of plasma cell differentiation from indirect effects. Whereas BMP-7 had limited direct effects on plasma cell differentiation, the other BMPs potently inhibited differentiation and BMP-6 was the strongest inhibitor. In contrast to BMPs, the role of TGF-β in Ig production is well studied. TGF-β directs IgA CSR in B cells [116], and can induce efficient IgA secretion if the cells are co-stimulated with for instance IL-10 [16]. However, the production of IgM and IgG is inhibited by TGF-β [117]. Furthermore, Activin A, another member of the TGF-β superfamily, has been reported to induce IgA [118] or IgG production [119] in mouse B cells. TGF-β and Activin A can also inhibit B lymphopoiesis by suppressing production of human $CD10^+$ B progenitor cells from primitive cord blood $CD34^+$ cells, whereas BMP-4 did not have any effect in this setting [120]. However, another study has shown that BMPs, including BMP-4, can regulate differentiation of $CD34^+$ cells [77], and BMP-6 is shown to inhibit production of $CD19^+$ B cells from $CD10^+$ B progenitor cells [121]. Overall, these studies together with paper I show the potency of TGF-β family members in controlling differentiation of B cells at different developmental stages, thereby regulating humoral immunity.

BMP-6 inhibited Th2 differentiation of naive $CD4^+$ Th cells (paper II). However, Noggin or soluble receptors (Alk2/Fc, BMPRII/Fc) were not able to counteract the BMP-6-induced effects, but instead inhibited Th2 differentiation themselves. Similarly, we experienced that

Noggin was unable to counteract the inhibitory effect of BMP-6 on Ig production in B cells, whereas counteracting with neutralizing anti-BMP-6 antibodies was successful (paper I). Note that neutralizing anti-BMP-6 antibodies were not tested in primary T cells. Furthermore, TGF-β was able to inhibit Th2 differentiation in naive $CD4^+$ T cells, and the effect was stronger than that observed for BMP-6 (paper II). TGF-β is known to inhibit differentiation from naive T cells to T effector cells, including acquisition of the cytotoxic function of $CD8^+$ cells and differentiation of $CD4^+$ cells to Th1 or Th2 cells [122]. BMPs have previously been shown to play a role in T-cell development as BMP-2 and BMP-4 are expressed in the thymus and BMP-4 inhibits differentiation from double negative to double positive T cells *in vitro* [123]. BMPs are also involved in murine thymus organogenesis [124]. Recently it was shown that BMP-2 and BMP-4 increased the ability of TGF-β to promote the generation of $Foxp3^+$ iTregs, both *in vivo* and *in vitro* [125]. However, this was not dependent on BMP signaling, and the BMPs were unable to substitute for TGF-β in iTreg induction. Activin A has also been shown to have synergistic effects on TGF-β-induced iTreg differentiation, and unlike BMPs, Activin A alone could promote iTreg differentiation [126]. Taken together, BMPs are able to affect differentiation of both B and T cells, suggesting an important immunoregulatory role of BMPs.

4.3.2 BMP-7 suppresses proliferation and induces apoptosis of normal B cells

In paper II, we showed that BMP-6 inhibited DNA synthesis in Jurkat TAg cells, but had no growth inhibitory effect in PHA- or anti-CD3/CD28-activated $CD4^+$ T cells. Survival was not affected in either cell types. It has previously been shown that BMP-2 and BMP-4 can inhibit proliferation of anti-CD3/CD28-activated murine $CD4^+$ T cells [127]. However, the BMP-4 effect was bimodular as lower doses were growth inhibitory and high doses had growth stimulating effects in the same cells [127]. Several doses of BMP-6 were tested in Jurkat TAg cells, and in these cells the inhibitory effect increased with increasing concentrations (paper II). TGF-β has been reported to inhibit proliferation in naive, but not activated $CD4^+$ T cells [128]. The unresponsiveness to TGF-β in activated T cells correlated with decreased expression of TGF-β receptor type II. Furthermore, TGF-β promotes survival during T cell expansion and differentiation [129].

In primary B cells, BMP-6 had some inhibitory effect on cell growth of CD40L/IL-21-stimulated memory B cells, but the growth inhibitory effect of BMP-7 was more potent in both naive and memory B cells (paper I). BMP-7 also potently induced cell death in both B cell populations whereas BMP-6 did not. In contrast, BMP-6 inhibited plasma cell differentiation directly whereas BMP-7 had limited effects (see section 4.3.1). The difference in functional outcome between these BMPs is surprising considering their structural similarity. Based on amino-acid structure, the BMPs are divided into subgroups, with BMP-2 and BMP-4 belonging to one group and BMP-5, -6 and -7 to another [73]. BMP-2/4 share 86% and BMP-6/7 share 71% amino acid identity. The different effects of BMP-6 and BMP-7 are also confirmed in paper III, where BMP-6 has a more potent inhibitory effect than BMP-7 in all the sensitive cell lines. BMP-2 and BMP-4 on the other hand had very similar effects in all functional assays performed in both normal and malignant B cells (paper I and III). This is in line with higher percentage of shared identity in BMP-2/4 than BMP-6/7. However, differential effects of BMP-2 and BMP-4 have been reported in the literature [77].

Our group has previously reported that BMP-6 inhibits proliferation of IgM-stimulated naive and memory B cells by 40% [78;121]. The inhibitory effect of BMP-6 on IgM-stimulated but low or lack of effect on CD40L/IL-21-stimulated B cells (paper I), suggests that the effect of BMPs depends on the co-stimulating conditions. B cell growth is also inhibited by TGF-β, as this cytokine inhibits proliferation and induce apoptosis in mature B cells [130;131]. In conclusion, the growth inhibitory effect of BMPs in normal B and T cells are variable and dependent on co-stimulating conditions. Of the BMPs tested in our system, BMP-7 was most potent at inhibiting DNA synthesis and inducing cell death.

4.4 BMP signaling and target genes

We used FACS to investigate receptor expression in normal and malignant B and T cells. The specificity of this method was tested with soluble receptors in paper II and has previously been confirmed by Western blotting [78]. The most striking similarity between the different lymphoid cells was high expression of the type I receptor Alk2 (paper I-III). The only lymphocyte population that did not express this receptor was unstimulated naive B cells (paper I). However, we showed that Alk2 expression could be induced by activating these cells. Of the other type I receptors, Alk3 was expressed by $CD4^+$ T cells, but not by mature B cells, whereas neither of them expressed Alk6 (paper I and II). Malignant B cells showed variable expression of Alk3 and Alk6 (paper III). B and T cells also differed in their expression of type II receptors. Whereas both normal CD4^{+} T cells and Jurkat TAg cells expressed BMPRII as their only type II receptor (paper II), ActRIIB was the only receptor that was expressed by both normal and malignant B cells (paper I and III). Of note, we previously detected low levels of Alk6 and BMPRII protein expression in CD19^{+} B cells by Western blot analysis [78], and mRNA expression of Alk2, Alk3, as well as all type II receptors has been detected in peripheral blood memory B cells [114]. This indicates that some receptors can be expressed below the FACS detection level. Despite differences in receptor expression between the lymphocyte populations, BMPs induced Smad1/5/8 activation in both B and T cells, showing that the receptors were expressed at a sufficient level and that they were functional (paper I and II). In T cells, we also observed activation of the MAPK pathways ERK1/2 and p38. Non-canonical BMP signaling pathways remain to be investigated in B-lineage cells.

In paper II, a microarray study was performed to identify BMP-6 target genes in Jurkat TAg cells. The highest ranked probesets were genes encoding Id1, Id2 and Id3, with 18-, 10- and 4.6-fold induction, respectively, after 2 hours of stimulation with BMP-6. Id1 was also strongly induced at the protein level as the expression level increased more than 6-fold. Furthermore, using Id1 siRNA, we partly counteracted BMP-6 induced suppression of proliferation in Jurkat TAg cells, indicating that Id1 was a mediator of the BMP-6 effects. Id1 has been shown to mediate BMP effects in other cells. For example, BMP-6 induced migration and tube formation via Id1 in endothelial cells [132]. We observed BMP-6-induced upregulation of Id1-3 mRNA in CD40L/IL-21-stimulated memory B cells as well (paper I), with Id1 showing the highest fold change (more than 6-fold upregulation). Based on this, we suggested Id1 as mediator of BMP-6 effects in memory B cells, although we were not able to formally verify this due to lack of optimal transfection methods for primary B cells (see section 4.1.4). The importance of BMP-induced effects, possibly mediated via Id1 induction, has previously been observed in our lab, as BMP-6 induced expression of Id1 mRNA and protein in pre-B and mature B cells, in which BMP-6 inhibited proliferation [78;121]. Id proteins are negative regulators of both B- and T-cell development through their inhibition of E proteins, a group of the basic helix-loop-helix family of transcription factors [133]. Id3 induced apoptosis and growth arrest in murine B progenitor cells [134], and in transgenic mice where T cells overexpressed Id1 or Id2, the T-cell development was blocked [135;136]. In mature B cells, overexpression of Id1, Id2 or Id3 inhibited CSR [137;138]. This is possibly due to reduced AID expression, as Id2 and Id3 can inhibit AID expression and E proteins can induce AID expression directly [26;139]. Furthermore, a defect in BCR-induced proliferation has been seen in Id3 knock out mice, leading to impaired humoral immune response [140]. Altogether, these studies together with our observations in paper I and III, points to a crucial role of BMPs to influence differentiation, proliferation and survival of B and T cells at various stages of development via their capacity to modulate the expression of Id proteins, in particular Id1.

Another gene that was regulated by BMP-6 in memory B cells was XBP-1 which is required for the secretory phenotype of plasma cells (paper I). BMP-6 significantly inhibited CD40L/IL-21-induced upregulation of XBP-1. It is not known if Id proteins or other BMP targets can regulate the transcription of XBP-1. In contrast, PRDM1, the gene encoding Blimp-1 which is also crucial for plasma cell differentiation, was not affected by BMP-6 in our study. However, this gene has been associated with some of the target genes from the microarray study in Jurkat TAg cells. Here, BMP-6 stimulation reduced the expression of Early growth response 1 (EGR1) and induced expression of NFKBIA, the gene encoding for IκBα which is a negative regulator of NF-κB (paper II). NF-κB signaling is shown to be necessary for PRDM1 induction in mice [141], and in humans, transcription of PRDM1 depends on a GC-box in the promoter binding EGR1 in addition to other transcription factors [142]. Furthermore, EGR1 is one of the proteins that are upregulated when CLL cells in mice are stimulated to differentiate to a plasma cell phenotype [143]. Another BMP-6 target gene found in the microarray study in Jurkat TAg cells was KFL10/TIEG (paper II) which is a known TGF-β target gene. In a study on pre-B cells, this gene has been shown to mediate the growth inhibitory effect of BMP-6 [144]. Taken together, these studies link several BMP-6 target genes to the inhibitory effects of BMPs we observed in B cells.

4.5 Role of BMP signaling in lymphomas

4.5.1 B-cell lymphomas can escape BMP-induced growth inhibition

BMP-2, -4 and -6 inhibited DNA synthesis in several B-cell lymphoma cell lines (paper III), which is in line with previous studies [78;145]. BMPs are also reported to inhibit proliferation and induce apoptosis in myeloma cell lines and primary samples from multiple myeloma patients [100-102;114;146], and TGF-β is shown to inhibit cell growth in B-cell lymphoma cell lines [147-149]. However, some B-cell lymphoma cells showed reduced sensitivity to BMP-induced growth inhibition. In fact, 3 out of 10 cell lines were resistant to the growth inhibitory effects of BMPs and in 3 out of 5 primary lymphoma samples, BMPs did not induce activation of Smad1/5/8 (paper III). We could not see any correlation between lymphoma type and sensitivity to BMPs as both sensitive and resistant cell lines and patient samples were found in all subtypes included. Loss of BMP responsiveness has previously been reported in several other cancers [150-152]. For example, a study reported that the BMP signaling pathway is inactivated, as judged by lack of nuclear pSmad1/5/8 expression in 70% of sporadic colorectal cancers [153].

In contrast to the inhibitory effects of BMPs in many cell lines, BMP-2 induced DNA synthesis in ROS-50 cells (paper III). This is also in line with previous studies where BMP-2 was shown to induce growth in several types of cancer [154-156]. Grčević et al. showed that BMPs could protect myeloma cell lines from apoptosis induced by an anti-myeloma drug (bortezomib) or TNF-related apoptosis-inducing ligand (TRAIL) [115], and BMPs are reported to enhance invasion and metastasis in malignant melanoma and breast cancer [157;158]. Differences in BMP response within the same cancer type, as we have seen in lymphoma cell lines, is not uncommon [159;160], and highlights the importance of investigating more than one cell line when the aim is to determine the role of BMPs in a certain type of cancer.

4.5.2 Possible mechanisms for BMP resistance

To determine the mechanism for how some B-cell lymphomas escaped the growth inhibitory effects of BMPs, we searched for alterations in the BMP signaling pathway. As a first approach, we compared BMP induced inhibition of DNA synthesis and activation of Smad1/5/8 for each cell line, and found a positive correlation between the two parameters. From this, we concluded that the mechanism of resistance often occurs early in the BMP signaling pathway, upstream of Smad1/5/8 activation. Additional mechanisms downstream of Smad1/5/8 might also contribute to BMP resistance as one of the resistant cell lines showed strong pSmad1/5/8 expression upon BMP stimulation.

We did not find downregulation of BMP receptors in lymphoma cell lines and patient samples (paper III). This was surprising as loss of receptors is a common mechanism of resistance in other cancer types. In this respect, loss of BMP receptor type II has been associated with BMP resistance in several types of cancer [150-153], and loss of TGF-β receptor expression has been shown in B- and T-cell lymphoma cells that are resistant to TGF-β-induced growth inhibition [149;161-165]. In some of these studies, BMP and TGF-β sensitivity could be restored by reintroducing the receptor [149;150;152;163]. We have not looked for mutations in the BMP receptors and thus cannot exclude that mutations, leading to truncated non-functional proteins which are still recognized by the antibodies, can be a mechanism of resistance in lymphoma. Furthermore, expression of BMP antagonists by tumor cells or cells in their microenvironment has previously been associated with BMP resistance in melanoma cells [166] and basal cell carcinoma [167]. However, we did not detect expression of BMP antagonists in lymphoma cell lines. Taken together, B-cell lymphoma cells develop other ways than loss of receptor expression or overexpression of antagonists to escape the negative influence of BMPs.

As B-cell lymphoma cells did not show reduced BMP receptor levels or enhanced antagonist production, we further investigated signaling pathway molecules downstream of the receptors. One of the resistant cell lines expressed low levels of Smad1 and Smad5, suggesting that downregulation of R-Smads can be a mechanism of resistance in this cell line (paper III). Interestingly, recent studies have linked expression of microRNAs (MiRs) to reduced BMP sensitivity through downregulation of R-Smads. In an EBV-negative Burkitt lymphoma cell line, MiR-155 has been shown to inhibit the expression of Smad1 and Smad5, thereby suppressing BMP signaling [168]. In the same study, the functional effects of BMPs, including growth inhibition, were reduced by MiR-155 [168]. In DLBCL cell lines, expression of MiR-155 is shown to limit the cytostatic effect of BMPs through direct suppression of Smad5 but not Smad1 [145]. Furthermore, MiR-155 has been suggested to have a role in lymphomagenesis as it is highly expressed in some lymphomas, particularly in the aggressive ABC subtype of DLBCL [169-172]. Additionally, Smad1 and Smad8 are downregulated in several types of cancer [173-175], and mice with a gonade specific deletion of Smad1/5 develop ovarian or testicular cancer [176], indicating a tumor suppressive role of R-Smads. However, overexpression of Smad1 has been reported in Follicular lymphoma [177;178] and Burkitt lymphoma [179] compared to normal B cells. In conclusion, downregulation of R-Smads could represent a common way to escape BMP-induced growth suppression, although high expression of R-Smads has also been reported in lymphomas.

Upregulation of inhibitory Smads represents another possible resistance mechanism as one resistant cell line expressed high levels of Smad7 mRNA, compared to sensitive cell lines (paper III). Furthermore, Smad7 was highly expressed in FL and DLBCL patient samples compared to CLL (paper III). Enforced expression of Smad7 inhibits TGF-β-induced growth inhibition in several cell lines, including a mouse immature B lymphocyte cell line [180-182]. Similarly, downregulation of endogenously expressed Smad7 restored TGF-β sensitivity in T-lymphoid cell lines [183]. Expression of Smad7 can also induce tumorigenicity in cancer cell lines [180;182]. Furthermore, it is suggested that expression of inhibitory Smads in human B-cell lymphoma cell lines contributes to autocrine TGF-β resistance [148]. In this aspect, expression of inhibitory Smads correlates with poor prognosis in lung cancer [184] and gastric carcinomas [185]. Taken together, several studies report a role for Smad7 in TGF-β resistance, and our data suggest a similar role for Smad7 in BMP resistance in lymphomas.

As seen in paper II, BMPs can also activate non-Smad signaling like MAPK-pathways, and crosstalk is seen between Smad signaling and other pathways (introduced in section 1.3.1). In lung cancer, interaction between BMP and Ras/MAPK signaling has been observed as epigenetic silencing of BMPs are associated with mutation in the k-ras gene, leading to aberrant Ras/MAPK pro-growth signaling [186]. Recent studies in multiple myeloma and lymphoma cell lines have shown that the antiproliferative effect of BMP-2 and TGF-β, respectively, depend on p38 MAPK signaling [146] (Bakkebø et al., submitted paper). Based on these results, it would be interesting to test if alterations in the p38 MAPK pathway, leading to inhibition of p38 MAPK signaling, also could contribute to BMP resistance in lymphomas.

4.5.3 Role of BMP-7 in lymphomagenesis

B-cell lymphoma cell lines and primary lymphoma samples expressed BMP-7 mRNA (paper III). The expression levels were high in all three tumor samples from FL patients, whereas samples from the more aggressive subtype DLBCL expressed low levels of BMP-

7. This finding involved few patients and needs confirmation in larger datasets, but suggests that BMP-7 expression can be inversely associated with tumor aggressiveness in B-cell lymphomas, with high expression in indolent tumors. Similarly, BMP-7 expression is reported to be inversely related to tumorigenic and metastatic potential in prostate and breast cancer [187;188]. However, BMP-7 expression is also found in bone metastatic tissue from prostate cancer and has been associated with accelerated bone metastasis formation in breast cancer [189;190].

BMP-7 mRNA was also expressed by normal B cells (paper III), and the expression in both normal and malignant B cells indicates that BMP-7 exists in the lymphoma microenvironment. The strong BMP-7-induced growth inhibition in normal B cells (paper I), points to an autocrine effect of BMP-7 in B cells. In line with this, Hsu et al. showed that virally induced BMP-7 had an autocrine growth inhibitory effect on normal melanocytes and primary/less aggressive metastatic melanoma cells [166;191]. Furthermore, aggressive melanomas were partly or completely resistant to BMP-7 [166]. Similarly, all lymphoma cells in our study showed low or no sensitivity to this BMP (paper III). To conclude, our data suggest that lymphoma cells gain a growth advantage compared to normal B cells by escaping the autocrine growth inhibition by BMP-7.

4.5.4 BMP-6 expression and correlation with survival

We found weak expression of BMP-6 mRNA in some lymphoma cell lines and malignant B cells from tumor samples (paper III). Expression of BMP-6 has previously been shown to correlate with aggressiveness and survival in B- and T-cell lymphomas. High BMP-6 expression was found to correlate with poor prognosis in a study of DLBCL [64], although this could not be verified in a later study in DLBCL treated with the CD20 monoclonal antibody Rituximab in combination with chemotherapy (R-CHOP) [192]. Promoter methylation of the BMP-6 gene has been reported to be frequent in aggressive subtypes of B-cell lymphomas [112] and aggressive forms of T-cell leukemia [193], and to correlate with decreased disease-free and overall survival in DLBCL [112]. Furthermore, the methylation of BMP-6 was correlated with decreased BMP-6 mRNA and protein expression in both studies [112;193]. High expression of BMP-6 has been correlated with poor prognosis in multiple myeloma patients [114]. Taken together, contrasting results are reported regarding the association between BMP-6 gene expression and prognosis, and the exact role of this BMP in lymphomagenesis warrants further investigations.

4.5.5 BMP signaling as therapeutic target in cancer

In the previous sections, the anti-tumoral effects of BMPs have been discussed and examples have been presented where B-cell lymphomas and other cancer types have lost sensitivity to BMPs and become resistant. In both situations, BMP signaling can be exploited as a therapeutic target in cancer therapy. Finding mechanisms for BMP resistance would open for treatment to regain BMP sensitivity, and in cancers were BMPs reduce growth, the BMPs themselves have therapeutic potential. Obviously, it would be important to determine which cancers are resistant and which are sensitive to BMPs. The situation is further complicated as BMPs can induce tumor growth and invasiveness in some tumors.

In lymphoma cells we found that potential BMP resistance mechanisms were downregulation of R-Smads and/or upregulation of inhibitory Smads. Possible forms of therapy to restore BMP sensitivity would be to re-introduce R-Smads, for instance by using recombinant R-Smads, or to reduce inhibitory Smad expression by use of selective small molecular inhibitors or antisense oligonucleotides. Xenograft models could represent the next step to investigate if these forms of therapy might work *in vivo*.

BMPs have been used to promote bone healing in orthopaedic practice. Various BMP-2- and BMP-7-based compounds are commercially available, and clinical studies have given promising results [194]. Importantly, there have been few reports of adverse events and little evidence for toxic side-effects. Considering the widespread effects of BMPs in various tissues, it is rather surprising that therapeutic use of BMPs do not have more side effects. We have for instance shown that BMPs have immunoregulatory effects (paper I and II). The lack of side-effects might be due to the presence of highly complex auto-regulatory systems that block BMP actions at various levels.

Since TGF-β has been shown to promote cancer progression, researchers have tried to develop TGF-β inhibitors for therapeutic use. Three main strategies for inhibition has been explored: i) compounds that interfere with binding of ligand to receptors ii) drugs that block intracellular signaling, for instance small molecule inhibitors and iii) antisense oligonucleotides [195]. Several of the compounds developed have shown potent anti-metastatic effects and inhibited tumor growth. One of the greatest risks in using TGF-β inhibitors is the potential acceleration of pre-neoplastic cancers in which TGF-β still functions as a tumor suppressor. However, this problem has not been observed *in vivo*. Another issue is the possibility for autoimmune phenomena and inflammations as seen in TGF-β knock-out mice. Since this has not been a problem in patients, it indicates that some TGF-β function is preserved despite the inhibitors.

Taken together, clinical use of BMPs and TGF-β inhibitors has shown promising results and few side-effects have been reported. This opens for the use of BMP signaling as a target for cancer therapy which needs to be further explored for B-cell lymphomas.

Conclusion

In this study we showed that BMPs were expressed by lymphocytes and that they affected several cellular functions in human B and T cells. Furthermore, Id proteins, in particular Id1, were BMP target genes which are likely to mediate the BMP effects. In more detail, we can draw the following conclusions:

- BMP-6 and BMP-7 mRNA were expressed by germinal center B cells and malignant B lymphoma cells, whereas T cells expressed low amounts of BMP mRNA.

- Normal and malignant B and T cells expressed BMP type I and type II receptors, and Smad1/5/8 were activated upon BMP stimulation. The exception was resting naive B cells, which had undetectable levels of type I receptors, but upregulated Alk2 expression in response to CD40L/IL-21.

- BMP-6 inhibited DNA synthesis in the T-ALL cell line Jurkat TAg, but not in normal peripheral blood $CD4^+$ T cells and only weakly in mature B cells. BMP-6 upregulated Id1-3 mRNA in normal B and T cells, and in Jurkat TAg cells, Id1 siRNA counteracted the growth inhibitory effects of BMP-6.

- BMP-6 inhibited plasma cell differentiation in naive and memory B cells and differentiation of $CD4^+$ T cells into IL-4-producing Th2 cells. In memory B cells, BMP-6 inhibited CD40L/IL-21-induced upregulation of XBP-1, a crucial transcription factor for plasma cell development.

- BMP-7 potently suppressed DNA synthesis and induced apoptosis in naive and memory B cells, but had limited direct effects on plasma cell differentiation.

- BMP-2, -4, -6 and -7 inhibited CD40L/IL-21-induced Ig production in naive and memory B cells.

- Some lymphoma cell lines and malignant B cells from lymphoma patient samples were resistant to BMP-2, -4 and -6, whereas all lymphoma cells showed reduced sensitivity to BMP-7. The BMP resistance mechanism is located early in the BMP signaling pathway, and possible mechanisms are downregulation of receptor-regulated Smads or upregulation of inhibitory Smads.

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II

III

Resistance to bone morphogenetic protein-induced growth inhibition in B-cell lymphoma

Kanutte Huse[1,2], Maren Bakkebø[1,2], Morten P. Oksvold[1,2], Sébastien Wälchli[1], Vera I. Hilden[1,2], Lise Forfang[1,2], Knut Liestøl[2,3], Erlend B. Smeland[1,2] and June H. Myklebust[1,2]

[1]Department of Immunology, Institute for Cancer Research, Oslo University Hospital; [2]Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo; [3]Department of Informatics, University of Oslo, Oslo, Norway

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Corresponding author: Dr. June H. Myklebust, Department of Immunology, Institute for Cancer Research, Oslo University Hospital HF, The Norwegian Radiumhospital, Postboks 4953 Nydalen, 0424 Oslo, Norway. E-mail address: june.helen.myklebust@rr-research.no

Abstract

Alterations in bone morphogenetic protein (BMP) signaling and BMP expression have been reported in some solid cancers and myeloma, but there are few studies on malignant lymphoma. Here we elucidated the functional influence of BMP-2, -4, -6 and -7 in human B-cell lymphomas. BMP mRNAs, in particular BMP-7 were detected in normal germinal center B cells as well as in malignant B cells. Adding exogenous BMPs to 10 lymphoma cell lines resulted in up to 90% inhibition of DNA synthesis in some cell lines, whereas 3 out of 10 were completely resistant. Importantly, BMP-7 had no or limited effect in all the lymphoma cells tested. Also, tumor samples from 3 out of 5 lymphoma patients were resistant to BMPs, as determined by lack of Smad1/5/8 activation. Sensitive and resistant cell lines were further compared to identify the cause of BMP resistance. We found a positive correlation between activation of Smad1/5/8 and inhibition of DNA synthesis. Of the three resistant cell lines, one was found to express high levels of inhibitory Smad7 mRNA and one had low levels of Smad1 and Smad5. Analysis of gene expression data confirmed high expression of Smad7 in lymphoma patient samples. Downregulation of BMP receptors is seen in many cancers, but we found similar receptor levels in resistant compared to sensitive lymphoma cells. In conclusion, we found that B-cell lymphomas expressed BMP-6 or BMP-7 mRNA, but could escape BMP-induced effects. Furthermore, BMP resistance correlated with reduced Smad1/5/8 activation, suggesting that the alterations often occur early in the BMP signalling pathway.

Introduction

Bone morphogenetic proteins (BMPs) are members of the TGF-β family of cytokines and control cellular processes like proliferation, apoptosis, migration and differentiation in many cell and tissue types (1). BMPs play important roles during embryonic development. They also regulate tissue homeostasis in adults, including hematopoietic stem cells, various stages of B-cell lymphopoiesis, and mature B cells (2-4).

BMP members transduce their signal via two types of serine/threonine receptors which they bind with different affinities (1). Type II receptors are constitutively active, whereas type I receptors require ligand binding, ligand-receptor oligomerization and transphosphorylation via type II receptors to be activated. The active type I receptors then phosphorylate the receptor Smads (R-Smads): Smad1, Smad5 or Smad8, which together with Smad4 form a complex and move to the nucleus where they bind DNA and regulate transcription of target genes. The pathway is negatively regulated on multiple levels, including intracellular inhibitory Smads: Smad6 and Smad7, and extracellular antagonists like noggin (5).

In several types of cancer, alterations in components of the BMP signaling pathway have been found, demonstrating their importance during tumorigenesis (6). Whereas some studies showed that BMPs can promote tumorigenesis and metastasis, others demonstrated that BMPs can have negative effects on cancer (7;8), indicating that BMP effects are highly context-dependent. TGF-β has been more extensively studied than BMPs, and is well established as a tumor suppressor. Tumor cells can evade the anti-tumoral effect of TGF-β by acquiring alterations in the TGF-β signaling pathway, such as mutations in receptors or Smad4 and upregulation of inhibitory Smads (9). Resistance to BMPs has also been shown in some cancers and the mechanisms for resistance are similar to those found in the TGF-β pathway (6). For instance, impaired expression of BMP receptors and Smad4 has been found in colorectal cancer (10). In this study we have investigated the functional response of BMPs, and we have studied the intracellular signaling proteins of BMPs in human B cell lymphomas.

Materials and Methods

Patient samples

Tumor biopsies were obtained from patients with follicular lymphoma (FL, $n = 6$) or *de novo* diffuse large B cell lymphoma (DLBCL, $n = 2$) at the Norwegian Radium Hospital between 1988 and 1993 (11). Biopsies were obtained with informed consent in accordance with the Declaration of Helsinki and the regional Committee for Medical Research Ethics, Region Eastern Norway. Single cell suspensions were prepared and stored in liquid nitrogen until use.

Reagents and antibodies

The following biotinylated antibodies were from R&D Systems (MN): anti-ActRIA, anti-BMP-RIA, anti-BMP-RIB, anti-BMP-RII, anti-Act-RIIA and anti-Act-RIIB. Goat serum was purchased from Sigma-Aldrich (MO) and Streptavidin-PE from Dako (Denmark). Anti-CD38-PC5 were from Beckman Coulter (CA), anti-IgD-PE, anti-Igκ-APC, anti-Igλ-PE, anti-CD3-FITC and anti-CD10-FITC were from Dako and anti-CD3-Pacific Blue, anti-CD77-FITC, anti-CD20-PerCPCy5,5 and anti-Lap2 were from BD (CA), anti-pSmad1/5/8 and anti-Smad4 were from Cell Signalling Technology (MA), anti-Smad1 were from Upstate (Millipore, MA) and anti-PGK1 were from Abcam (MA). Recombinant human (rh) BMP-2 (300 ng/ml), rhBMP-4 (50 ng/ml), rhBMP-6 (500 ng/ml), rhBMP-7 (400 ng/ml), rhTGF-β (10 ng/ml), rhIL-2 (10 ng/ml), rhIL-4 (40 ng/ml) and rhIL-10 (10 ng/ml) were purchased from R&D Systems. CD40L (0.25 μg/ml) were purchased from Alexis Biochemicals (Enzo Life Sciences, NY). Dorsomorphin (Calbiochem, Merck, Germany) was used at 1.25 μM as this concentration inhibited the BMP induced effects without being toxic as determined by dose-response-experiments (data not shown).

Cell Culture

The cell lines Raji (purchased from DSMZ), Sudhl-4, Sudhl-6 (gift from L.M. Staudt, Metabolism Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD), K-422, ROS-50 (gift from J. Delabie, Dept of pathology, Oslo University Hospital, Oslo, Norway), Ramos and Bjab were cultured in RPMI 1640 (PAA Laboratories, Austria) supplemented with 10% fetal calf serum, penicillin and streptomycin. OCI-Ly7, OCI-Ly3 and OCI-Ly10 (gift from L.M. Staudt) were cultured in IMDM medium supplemented with 20% human plasma, 55 μM β-mercaptoethanol, penicillin and streptomycin. In all experiments, cells were cultured in serumfree X-VIVO15 medium (BioWhittaker, Switzerland).

FACS analysis and cell sorting

All incubations with antibodies were performed in the dark at 4 °C for 30 min and FACS analysis was performed on a FACSCalibur (BD) or LSRII (BD). Centrocytes and centroblasts were isolated from children undergoing routine tonsillectomy as described previously (12). Shortly, $CD19^{+}$ cells were isolated by immunomagnetic beads (CD19 Dynabeads, Invitrogen, CA) and stained with antibodies before $CD38^{+}IgD^{-}CD77^{-}$ centrocytes and $CD38^{+}IgD^{-}CD77^{+}$ centroblasts were FACS-sorted on a FACS Diva (BD). Tumor cell suspensions from lymphoma patients were stained with antibodies and subjected to FACS sorting into malignant B cells ($CD20^{+}CD10^{+}CD3^{-}Ig\kappa/Ig\lambda^{+}$) or infiltrating T cells ($CD20^{-}CD10^{-}CD3^{+}$).

Western blotting

Cells were lysed in SDS-lysis buffer (10 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 6.98% β-Mercaptoethanol, 1x protease inhibitor (Complete Mini, Roche, Switzerland) and 1x phosphatase inhibitor (PhosSTOP, Roche)). Nuclear fractions were made by using the Nuclear/Cytosol Fractionation Kit (BioVision, CA). Cell lysates were electrophoresed through 10% SDS-polyacrylamide gels (Pierce, IL) and transferred to PVDF membranes (Millipore, MA). The membranes were blocked for 60 min with 5% non-fat dry milk or 5% BSA (Sigma-Aldrich) in TBST before the specific antibodies were used. After washing in TBST, the membranes were incubated for 60 min with HRP-conjugated anti-rabbit or anti-goat IgG antibodies (Dako). Protein bands were visualized by the ECL or ECL-plus detection system (GE Healthcare, NJ). Densitometric analysis was performed by scanning films on a GS-800 Calibrated Densitometer (BioRad) and using Quantity One software (Bio-Rad, CA). Quantification of phosphorylated Smad1/5/8 levels across different western blots was obtained by normalizing to the positive control which was the same in each blot.

Proliferation assay

Cells were cultured in triplicates in 96 well round-bottom plates (20 000 cells/well in 200 µl) for 3 days and 20 µl of 3H-thymidine were added the 4 last hours of incubation. 3H-thymidin incorporation was measured as described previously (4).

Cell division tracking

Cells were labelled with 5 µM CFSE (Molecular Probes, OR) for 10 min at 37 °C and cultured over night before a narrow peak (about 10% of the population) of cells with the same CFSE intensity was sorted. The CFSE-sorted cells were cultured with or without BMPs for 3 days (20 000 cells/well in 200 µl) before FACS analysis of CFSE intensity was performed on a FACS Canto (BD).

Determination of cell death

Cells were cultured for 3 days and stained with 15 µg/ml propidium iodid (PI; Invitrogen) or TUNEL (Roche) according to the manufacturers' recommendations. The cells were analyzed on a FACS Calibur (BD).

Real-time RT-PCR and gene expression data

RNA was isolated from cells using the Absolute RNA miniprep kit (Stratagene, CA) following the manufacturer's recommendations. cDNA was synthesized and analyzed by real-time RT-PCR using TaqMan technology (Applied Bioscinces, CA), and gene expression was quantified using the comparative C_T method as previously described (3). PGK1 was used as endogenous reference and gene expression in each sample were normalized to the level in fetal brain tissue (BioChain, CA).

Smad mRNA expression data ($\log_2$ transformed) were analyzed across different lymphoma groups in the microarray dataset from Alizadeh et al. (13), and included samples from patients with Chronic lymphocytic leukemia (CLL; $n = 29$), DLBCL ($n = 42$) and FL ($n = 9$).

Statistical analysis

Statistical comparisons of groups were calculated using two-sided, paired Student's t-tests. In Fig. 2A the mean value of triplicates in each experiment was log-transformed before doing the t-test. The relation between BMP-induced phosphorylation of Smad1/5/8 and suppression of DNA synthesis were evaluated by analysis of covariance, using DNA synthesis and an indicator for the BMPs as factors in the analysis (JMP 7.0 software).

Results

Expression of BMP mRNA in normal and malignant germinal center B cells

The expression of BMPs in adult lymphoid tissue is largely unknown. Therefore, we wanted to examine the expression of BMP mRNA in normal and neoplastic germinal center B cells. We purified $CD19^{+}$ cells from tonsils from healthy donors and used FACS to sort centrocytes ($CD19^{+}CD38^{+}IgD^{-}CD77^{-}$) and centroblasts ($CD19^{+}CD38^{+}IgD^{-}CD77^{+}$). Using real-time RT-PCR, we found that these germinal center B cells expressed BMP-7, but only low levels of BMP-6 compared to fetal brain (positive control; Fig. 1A). Studies in lymphoma cell lines of different subtypes showed that 7 out of 10 expressed BMP-7, whereas 2 out of 10 had detectable BMP-6 levels (Fig. 1B). Only one cell line expressed BMP-4 (Supplementary Fig. S1), and BMP-2 mRNA was undetectable (data not shown).

Next, we used tumor samples from lymphoma patients and separated the malignant B cells ($CD20^{+}CD10^{+}Ig\kappa/Ig\lambda^{+}$) from the infiltrating T cells ($CD20^{-}CD3^{+}$) by FACS sorting. BMP-6 was expressed at low to intermediate levels in all malignant B cells, whereas infiltrating T cells expressed undetectable to low levels of BMP-6 and BMP-7 (Fig. 1C). Furthermore, there was a marked difference in BMP-7 expression between the two subtypes of lymphoma, with follicular lymphoma (FL) B cells expressing large amounts of BMP-7 whereas diffuse large B-cell lymphoma (DLBCL) cells did not express BMP-7. Altogether, the expression of BMP-6 and BMP-7 in normal as well as malignant germinal center B cells suggests the possibility for autocrine growth regulation.

B-cell lymphoma cells can escape BMP-induced inhibition of cell growth

We have previously shown that BMP-6 inhibits DNA synthesis in normal B cells (4). As lymphoma cells expressed BMP-6 and -7 mRNA (Fig. 1), we next studied the effects of exogenously added BMPs in different B-cell lymphoma cell lines. BMP-2, -4 and -6 induced more than 30% inhibition of DNA synthesis in three cell lines (Raji, Sudhl-6, OCI-Ly3) of which Sudhl-6 was most affected (Fig. 2A). These were defined as BMP sensitive. In contrast, three other cell lines (ROS-50, K-422, OCI-Ly7) were completely resistant to BMP-induced inhibition of DNA synthesis. Furthermore, four cell lines (Bjab, Ramos, Sudhl-4, OCI-Ly10) showed intermediate sensitivity with less than 30% inhibition for any BMP tested (Supplementary Fig. 2). Interestingly, sensitivity to BMP-7 was low in all cell lines, with less than 20% inhibition of DNA synthesis (Fig. 2A; Supplementary Fig. S2). In sensitive Sudhl-6 cells, CFSE tracking of cell division confirmed that proliferation was inhibited by BMP-2 and BMP-6 (Fig. 2B). Induction of cell death was less prominent, except for Sudhl-6 cells (Supplementary Table I and II). Altogether, B-cell lymphoma cell lines had variable sensitivity to BMP-2-, -4- and -6-induced growth inhibition, but they were all resistant to BMP-7.

Cytokines can counteract the inhibitory effects of exogenous BMP-6 in sensitive cells

The lymph node microenvironment normally contains many cytokines including IL-2, IL-4 and IL-10, mainly produced by activated T cells which also express membrane bound CD40L (14). Thus, we next tested if these B-cell stimulators could counteract the inhibitory effect of exogenous BMP-2 and -6 in sensitive Sudhl-6 cells. IL-10 and CD40L partly abolished the effect of BMP-2 and -6 by themselves, but the combination of these was more potent and increased DNA synthesis in BMP-6-stimulated cells from 11% to 46% (compared to unstimulated cells; Fig. 2C). In contrast, IL-4 or IL-2 did not counteract the effect of BMPs (Fig. 2C; data not shown). Taken together, IL-10 and CD40L could partly counteract the BMP-induced growth suppression in sensitive Sudhl-6 cells.

Sensitive and resistant lymphoma cells express BMP receptors, but limited levels of antagonists

Tumor cells and cells of their microenvironment have been shown to express BMP antagonists to protect against growth inhibitory effects of BMPs (15;16). We measured mRNA levels of several BMP antagonists in lymphoma cell lines. No detectable expression was observed for SOSTDC1, SOST, CHRDL1 and CHRDL2, whereas Noggin and Chordin mRNA were expressed at low levels by Ramos and K-422, respectively (data not shown, $n = 2$). Thus, upregulation of BMP antagonists seems not to be a common way for lymphomas to escape BMP-induced growth inhibition.

Next, we focused on how some lymphoma cells could escape BMP-induced growth suppression by comparing BMP-induced signal transduction in senstitive and resistant cell lines. Expression of BMP receptors are reduced in several types of cancer and this could be a mechanism to evade BMP-induced suppression of proliferation (10;17-19). We used FACS analysis to determine the expression of BMP receptors in lymphoma cell lines and primary lymphoma samples. The sensitive cell line OCI-Ly3 is shown as an example and expressed high levels of activin receptor-like kinase (Alk) 2, activin receptor type II

(ActRII) A and ActRIIB, and low levels of the other three receptors (Fig. 3A). All resistant cell lines expressed at least one type I and one type II receptor at comparable levels to sensitive cell lines as shown by median fluorescence intensity (MFI) relative to isotype control (Fig. 3B). In addition, the resistant cell line K-422 expressed high levels of receptors compared to sensitive cell lines.

We also included tumor samples from lymphoma patients, and stained the cells with anti-CD3, -CD20, -Igκ and -Igλ antibodies to specifically analyze BMP receptor expression in lymphoma cells. Malignant B cells from all patients expressed Alk2 and ActRIIB, although to a variable degree (Fig. 3C). Most of them also expressed Alk6 and ActRIIA. Furthermore, the expression of the various BMP receptors was not different from the normal B cells present in the same sample (Supplementary Table III). These results indicate that downregulation of receptors is not a common mechanism for loss of BMP sensitivity in lymphomas.

BMP-induced growth suppression correlates with induction of pSmad1/5/8

Next, we wanted to test if resistance to BMP is due to changes upstream or downstream of Smad1/5/8 activation. To optimize conditions, Western blots were performed to detect BMP-induced activation of Smad1/5/8 proteins in sensitive Sudhl-6 cells. Based on time course experiments (Supplementary Fig. S3A), a one-hour incubation period was selected for further studies of BMP-induced signaling. Furthermore, Dorsomorphin, a selective inhibitor of BMPs (20), completely abolished BMP-induced phosphorylation of Smad1/5/8 (Supplementary Fig. S3B) and growth inhibitory effects (Supplementary Fig. S3C), thus showing specificity of the BMPs. To normalize pSmad1/5/8 induction across the different lymphoma cell lines, a positive control of Sudhl-6 cells cultured with BMP-2 for 1 hour were used in each blot. Two out of three resistant cell lines showed very low or no phosphorylation in response to any of the BMPs tested (Fig. 4A). In resistant ROS-50 cells, BMP-2 and BMP-4 induced some activation of Smad1/5/8, but note that BMP-2 significantly increased the DNA synthesis in these cells (Fig. 2A). As expected, BMP-2 and -4 induced strong phosphorylation of Smad1/5/8 in the three sensitive cell lines (Fig. 4A). In most cell lines, except Sudhl-6, BMP-7 did not induce phosphorylation of Smad1/5/8 which is in agreement with no/limited inhibitory effects of this BMP. The correlation between BMP-induced phosphorylation of Smad1/5/8 and suppression of DNA synthesis in the various lymphoma cell lines was significant when all the different BMPs were combined ($p = 0.015$, adjusted R-square = 40%; Fig. 4B). Similarly to the variable level of BMP-induced phosphorylation of R-Smads in cell lines, analysis of BMP-induced phosphorylation in five lymphoma patient samples (3 FL and 2 DLBCL) showed that lymphoma cells from 2 patients were responsive whereas the 3 others were unresponsive (Fig. 4C). As in the cell lines, BMP-7 did not activated Smad1/5/8 in any of the patient samples. Note that the BMP receptor levels in the non-responsive samples were not different from those which showed induction of pSmad1/5/8 upon BMP-2, -4 and -6 stimulation (Fig. 3C and 4C). As this is similar to what we observed for lymphoma cell lines, we expect that primary lymphoma cells can escape the negative influence of BMPs also *in vivo*. Collectively, BMP-induced phosphorylation of Smad1/5/8 was correlated with the functional effects of BMPs in lymphoma cell lines, suggesting that BMP resistance mechanisms in lymphoma cells are upstream of R-Smad activation.

Downregulation of R-Smads and upregulation of inhibitory Smad7 are potential mechanisms for loss of BMP responsiveness

The expression of Smad1 and Smad5 varied between the cell lines. Whereas two of the resistant cell lines, ROS-50 and OCI-Ly7, expressed high levels of Smad1 and Smad5, K-422 cells expressed low levels of R-Smads (Fig. 5A and B). The sensitive cell lines expressed low levels of Smad1, but with the exception of OCI-Ly3, they expressed high levels of Smad5. As the resistant K-422 cells expressed lower levels of Smad1 and Smad5 than the other cell lines, this suggests downregulation of R-Smads as a mechanism for loss of BMP responsiveness in this cell line.

To test whether phosphorylation of Smad1/5/8 mediated nuclear translocation of Smad1/5/8 and Smad4, nuclear fractions were made of Sudhl-4 and Sudhl-6 cells stimulated with BMP-2 for various periods of time. We observed higher levels of phosphorylated nuclear Smad1/5/8 in BMP sensitive Sudhl-6 cells, compared to the less sensitive Sudhl-4 cells (Fig. 5C). In contrast, nuclear Smad4 was expressed at comparable levels and the expression levels did not change after stimulation, suggesting a constant shuttling of Smad4 between cytoplasm and nucleus independent of R-Smad activation status. Furthermore, although the total protein levels of Smad4 varied between cell lines, it did not correlate with sensitivity to BMPs (Fig. 5D). Of the resistant cell lines, only OCI-Ly7 expressed low levels of Smad4, but the levels were not lower than in BMP-sensitive Sudhl-6 cells.

Using real-time RT-PCR, we next investigated if resistant cells expressed higher levels of inhibitory Smads. Whereas all cell lines expressed low levels of Smad6, BMP resistant ROS-50 cells expressed higher levels of Smad7 than sensitive cell lines (Fig. 6A). Thus, upregulation of Smad7 could be the mechanism of resistance in ROS-50. Gene expression profiling in CLL, FL and DLBCL showed that FL and DLBCL had high expression of Smad7 compared to CLL, suggesting that these lymphomas overall might have reduced sensitivity to BMPs (Fig. 6B). The expression of Smad6 was lower in FL than DLBCL, whereas Smad4 and Smad1 were expressed at similar levels. Altogether, we have seen that B-cell lymphomas can upregulate inhibitory Smad7 or downregulate R-Smads and these events represent possible mechanisms for resistance to BMPs.

Discussion

BMPs are known to inhibit proliferation and induce apoptosis in many types of cells, including B cells (4). In cancer, alterations have been found in several components of the BMP signaling pathway, leading to BMP resistance. In this study, we have shown that B-cell lymphoma cells as well as normal germinal center B cells express BMP mRNA, most frequently BMP-7. Strikingly, we observed that all lymphoma cells were resistant to this BMP whereas their sensitivity to BMP-2, -4 and -6 varied from highly sensitive to completely resistant. Our data suggests that more than one mechanism is involved in the resistance to BMPs, including downregulation of R-Smads and upregulation of inhibitory Smad7.

Both sensitivity and resistance to BMPs have been reported in the literature, which is in line with the variation we observed in sensitivity to BMPs. Some studies report that cancer cells, including hematopoietic malignancies, are resistant to BMPs (10;17;18;21), whereas other studies show that BMPs induce apoptosis and inhibit proliferation (4;22-24). In ROS-50 cells, we found that BMP-2 induced DNA synthesis, suggesting rewired signaling and induction of target genes different from those normally induced when BMPs have anti-proliferative effects. This is also in line with previous studies showing that BMP-2 can induce tumor growth under certain conditions (25-27). Sensitivity to BMP-7 was lower than to the other BMPs, both in cell lines and in lymphoma samples. Combined with expression of BMP-7 in both normal and malignant B cells, this suggests that lymphoma cells can escape autocrine growth inhibitory effects of BMP-7. Aggressive metastatic melanoma cells have previously been shown to be resistant to autocrine growth inhibitory effects of BMP-7 (16). The same study showed that BMP-7 expression correlated with tumor progression in melanoma cells, as aggressive melanomas expressed abundant BMP-7 mRNA and primary melanomas did not (16). Expression of BMP-6 has previously been associated with prognosis in patients with hematopoietic malignancies. In multiple myeloma patients, Seckinger et al. showed that high BMP-6 expression correlated with increased overall survival (28). In DLBCL, a study showed that hypermethylation of the BMP-6 promoter which correlated with lack of BMP-6 expression, was associated with decreased survival (29). However, a study by Rosenwald et al., where BMP-6 increased the prognostic value of a gene expression signature in DLBCL, showed that BMP-6 expression was associated with poor outcome (30). The finding that BMPs have a role in hematopoietic malignancies is further strengthened as Grčević et al. (31) showed that expression of BMP-4 and BMP-6 was significantly higher in bone marrow samples from multiple myeloma patients than from healthy control subjects.

Downregulation of BMP receptors has been shown in cancer, and lost sensitivity can be restored by exogenous expression of BMPRII (10;17;18). In a lymphoma cell line, it has been shown that resistance to TGF-β correlates with epigenetic silencing of TGF-β receptor II (32). However, we did not detect loss of BMP receptors in lymphoma cell lines or lymphoma patient samples. Furthermore, the BMP receptor expression in lymphoma cells did not differ from the non-malignant B cells in patient samples. Thus, escape from BMP-induced growth inhibition in B-cell lymphomas seems not to be mediated via downregulation of BMP receptors, unless there are mutations leading to truncated non-functional proteins that are still recognized by the antibodies. In this respect, mutations in Activin type II receptor and Alk3 has been found in gastrointestinal tumors and patients with juvenile polyposis, respectively (33-35). These mutations can lead to non-functional receptors, for instance receptors lacking kinase domains. As we have not looked for mutations in the BMP receptors, we can not exclude that mutations is a mechanism of resistance in lymphoma.

Although we found a statistical significant relationship between BMP-induced Smad1/5/8 activation and growth inhibition, BMPs induced Smad1/5/8 phosphorylation in one resistant cell line. This implies that the mechanism of resistance also can be found downstream of Smad1/5/8 phosphorylation, and that lymphoma cells can develop different ways to escape the negative influence of BMPs. Smad4 was originally identified as a tumor suppressor and deletions or mutations in Smad4 is common in solid cancers, including 50% of all pancreatic cancers (9). However, mutations in Smad genes are rare in hematopoietic tumors (36). All lymphoma cell lines in this study expressed Smad4 although at different levels, and Smad4 had similar expression levels in DLBCL, FL and CLL. Furthermore, most tumors with mutated Smad4 genes lack Smad4 expression, although some mutations lead to truncated proteins unable to bind other Smads or inhibiting DNA binding (6;37). However, we have no indications for existence of truncated Smad4 in the lymphoma cell lines we tested. In contrast, downregulation of R-Smads are more likely to contribute to BMP resistance, as Smad1 and Smad5 were less expressed in resistant K-422 cells than in the other cell lines. The low expression level of R-Smads in this lymphoma cell line correlated with very low/no induction of phosphorylation of Smad1/5/8. R-Smads have previously been shown to have a tumor suppressive role, as gonade specific deletion of Smad1/5 induces ovarian or testicular cancer in mice (38). It has been suggested that microRNA-155 has a role in lymphomagenesis as it is highly expressed in some lymphomas (39;40). MicroRNA-155 expression can lead to limited cytostatic effect of BMPs through direct suppression of Smad5 (21), and has been shown to be overexpressed in the aggressive ABC subtype of DLBCL (21;40;41). Hence, downregulation of Smad5 could represent a common way to escape BMP-induced growth suppression.

Upregulation of inhibitory Smads represents another mechanism of BMP resistance as resistant ROS-50 cells expressed high levels of Smad7 mRNA compared to sensitive cell lines. This combined with the overall high expression of Smad7 in FL and DLBCL samples compared to CLL, suggests Smad7 as an important mediator of BMP resistance in lymphomas. Smad6/7 inhibit BMP signaling in multiple ways, including competition of binding to activated receptors or Smad4 (5). In addition, Smad7 can inhibit Smad-signaling in the nucleus by disrupting the formation of functional Smad-DNA complexes (42). Smad6/7 are upregulated in pancreatic cancer (43;44) and correlates with poor prognosis in lung cancer (45) and gastric carcinomas (46). It has also been shown that Smad6/7 is expressed in lymphomas (47).

In addition to alterations in the BMP signaling pathway, cytokines normally present in the lymph node environment could influence the functional outcome of BMP signaling. We found that the inhibitory effect of BMPs could be reduced by adding CD40L and IL-10. Similarly, it has previously been shown that stimulation through CD40 inhibits TGF-β effects through induction of Smad7 (48). Interestingly, production of IL-10 has previously been observed in Non-Hodgkin lymphomas and IL-10 can induce proliferation of these tumor cells (49). It has also been shown that elevated IL-10 plasma levels are associated with poor prognosis in DLBCL (50). Since CD40L and IL-10 are present in the lymphoma microenvironment, these proteins can contribute to the BMP resistance.

In conclusion, we have shown that some B-cell lymphomas can escape BMP-induced anti-proliferative effects and that this correlates with reduced Smad1/5/8 activation. BMP resistance can be mediated via downregulation of R-Smads or upregulation of inhibitory Smads, whereas loss of BMP receptors was not seen in lymphomas. How loss of BMP sensitivity might influence lymphomagenesis warrants further investigations, for example by using xenograft models.

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Figure legends

Figure 1

Normal and malignant B cells express BMP-6 and BMP-7. BMP mRNA expression was determined by real-time RT-PCR, and the results are normalized to the expression in human fetal brain. A, centrocytes ($CD19^{+}CD38^{+}IgD^{-}CD77^{-}$) or centroblasts ($CD19^{+}CD38^{+}IgD^{-}CD77^{+}$) from normal human tonsils (relative expression ± SD of duplicates). B, lymphoma cell lines (means ± SEM, $n = 3$). C, purified malignant B cells ($CD20^{+}CD10^{+}Ig\kappa/Ig\lambda^{+}$) or tumor-infiltrating T cells ($CD20^{-}CD3^{+}$) from lymphoma patient samples (relative expression ± SD of duplicates).

Figure 2

Effects of BMPs on proliferation of B-cell lymphoma cell lines. A and C, lymphoma cell lines were stimulated with various BMPs alone (A; $n = 6$, * $p < 0.05$) or in the presence of interleukins and CD40L (C; $n > 2$) for three days before measuring 3H-thymidine incorporation. Results are normalized to unstimulated control in each experiment. B, tracking of cell division by CFSE labelling of Sudhl-6 cells cultured with or without BMP-2 or BMP-6 for 3 days ($n = 4$).

Figure 3

BMP sensitive and resistant lymphoma cells express BMP receptors. A, BMP receptor expression in OCI-Ly3. B and C, relative receptor expression in lymphoma cell lines (B; means ± SEM, $n = 3$) and $CD20^{+}CD3^{-}Ig\kappa/Ig\lambda^{+}$ malignant B cells from lymphoma patient samples (C). Values represent median fluorescent intensity (MFI) of each BMP receptor relative to the MFI of the isotype control.

Figure 4

Reduced phosphorylation of Smad1/5/8 in resistant lymphoma cells. A, lymphoma cell lines were stimulated with BMPs for one hour and analyzed for the expression of pSmad1/5/8 by Western blotting. B, BMP-induced phosphorylation of Smad1/5/8 was quantified and mean OD-values ($n = 3$) are plotted against values for relative DNA synthesis (from Fig. 2A) for each cell line. The parallel lines are based on analysis of covariance; this analysis shows a significant correlation ($p = 0.015$) between the BMP-induced phosphorylation and the relative DNA synthesis. C, tumor samples from five different lymphoma patients were stimulated with BMPs for one hour and analyzed for the expression of pSmad1/5/8. Anti-PGK1 was used as loading control and vertical lines indicate cutting of gel.

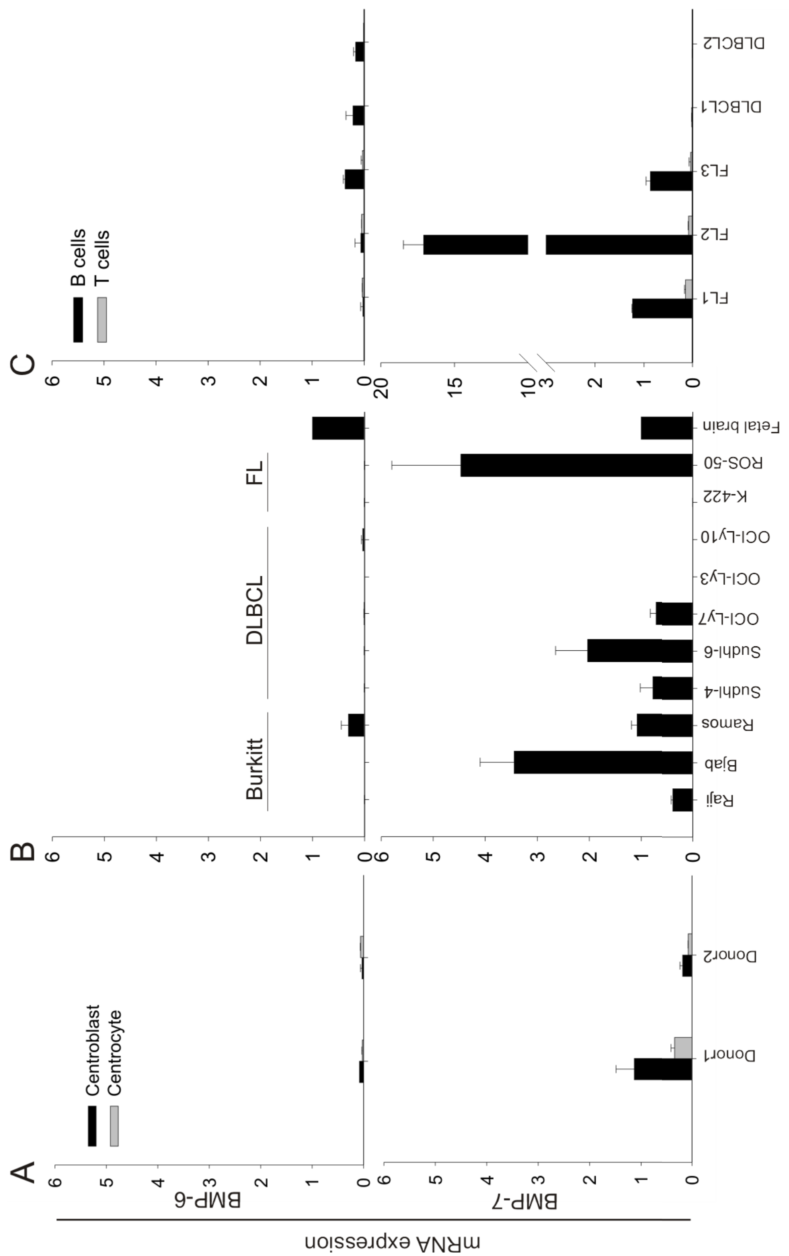
Figure 5

Expression of R-Smads and Smad4 in lymphoma cell lines. A and B, Smad1 and Smad5 expression was measured by Western blotting in cell lines. Anti-PGK1 was used as loading control. One representative experiment (A) and mean values of optical density (OD) from densitometric measurements (B; ± SEM, $n = 3$). C, Sudhl-4 and Sudhl-6 cells were stimulated with BMP-2 for different periods of time before nuclear fractions were made and analyzed for pSmad1/5/8 and Smad4 expression by Western blotting. Anti-Smad5 or anti-LAP2 was used as loading control. D, Smad4 expression in unstimulated cells, analyzed by Western blotting. Anti-PGK1 was used as loading control.

Figure 6

Expression of inhibitory Smads in lymphoma cells. A, Real-time RT-PCR analysis measuring Smad6 and Smad7 expression in cell lines. Data are given relative to the expression of Smad6 and Smad7 in human fetal brain. (Means ± SD, $n = 2$) B, Smad mRNA expression data ($\log_2$ transformed) analyzed across different lymphoma groups in microarray dataset from Alizadeh et al. (13).* $p < 0.0001$ compared to CLL.

Figure 1


A
B
C
Centroblast
Centrocyte
B cells
T cells
Burkitt
DLBCL
FL
BMP-6
BMP-7
mRNA expression
Raji
Bjab
Ramos
Sudhl-4
Sudhl-6
OCI-Ly7
OCI-Ly3
OCI-Ly10
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ROS-50
Fetal brain
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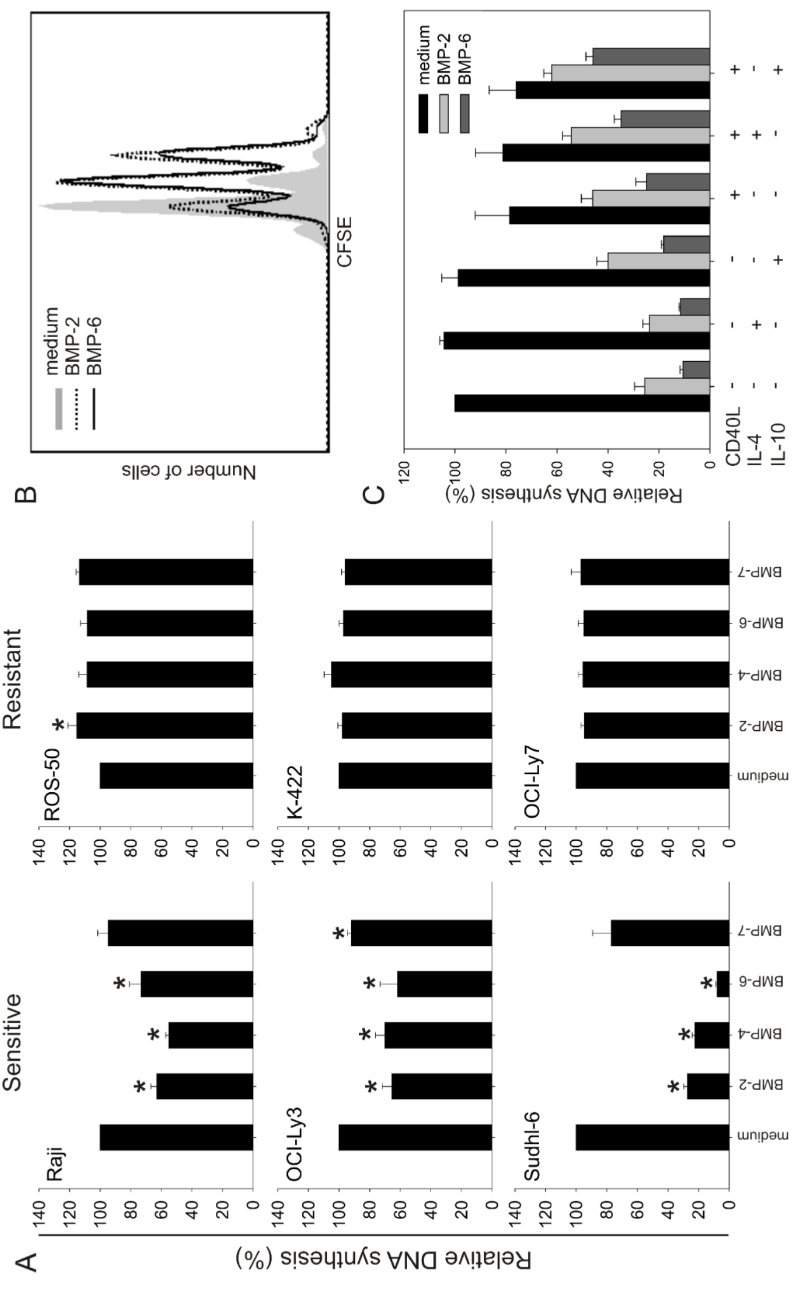
Donor2
FL1
FL2
FL3
DLBCL1
DLBCL2

Figure 2


A
Sensitive
Resistant
Relative DNA synthesis (%)
Raji
OCI-Ly3
Sudhl-6
ROS-50
K-422
OCI-Ly7
medium
BMP-2
BMP-4
BMP-6
BMP-7
B
Number of cells
CFSE
medium
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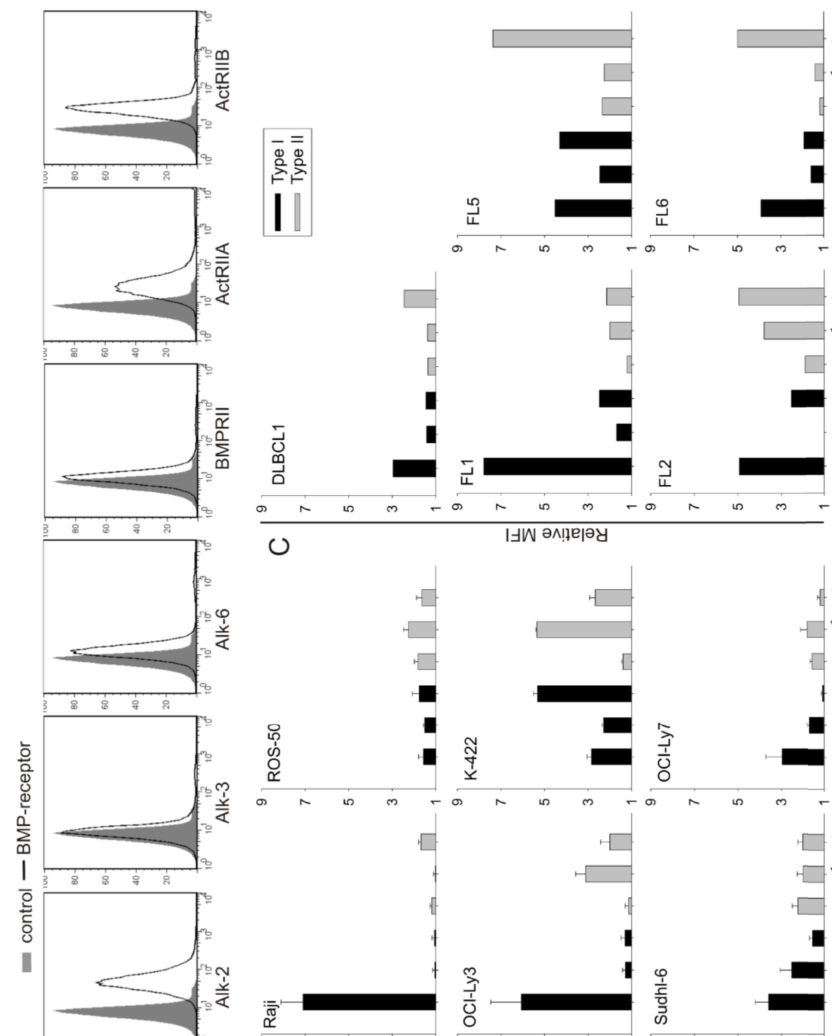
C
Relative DNA synthesis (%)
CD40L
IL-4
IL-10

Figure 3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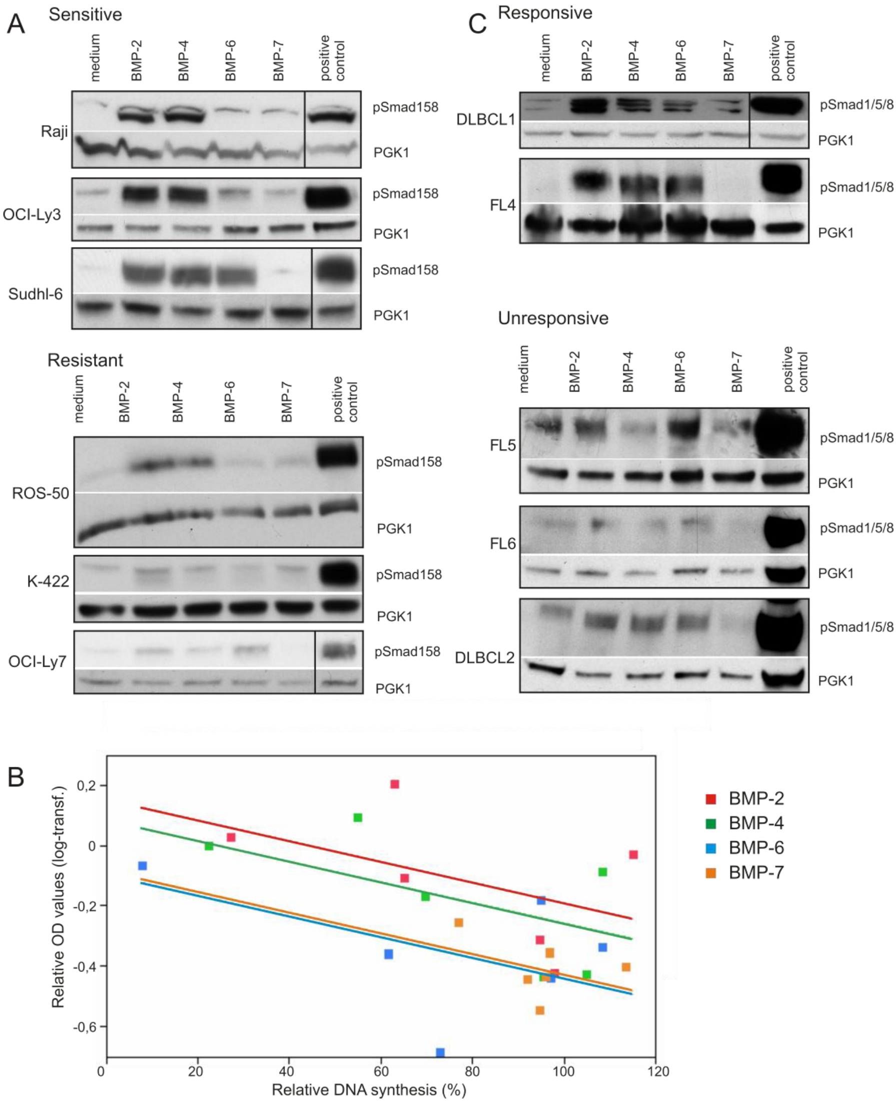
control
BMP-receptor
counts
Alk-2
Alk-3
Alk-6
BMPRII
ActRIIA
ActRIIB
B
Relative MFI
Raji
OCI-Ly3
Sudhl-6
ROS-50
K-422
OCI-Ly7
C
Relative MFI
DLBCL1
FL1
FL2
FL5
FL6
Type I
Type II
AlK-2
AlK-3
AlK-6
BMPRII
ActRIIA
ActRIIB

Figure 4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BMP-7
positive control
Raji
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PGK1
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pSmad158
PGK1
Sudhl-6
pSmad158
PGK1
Resistant
ROS-50
pSmad158
PGK1
K-422
pSmad158
PGK1
OCI-Ly7
pSmad158
PGK1
C
Responsive
DLBCL1
pSmad1/5/8
PGK1
FL4
pSmad1/5/8
PGK1
Unresponsive
FL5
pSmad1/5/8
PGK1
FL6
pSmad1/5/8
PGK1
DLBCL2
pSmad1/5/8
PGK1
B
Relative OD values (log-transf.)
0,2
0
-0,2
-0,4
-0,6
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40
60
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100
120
Relative DNA synthesis (%)
BMP-2
BMP-4
BMP-6
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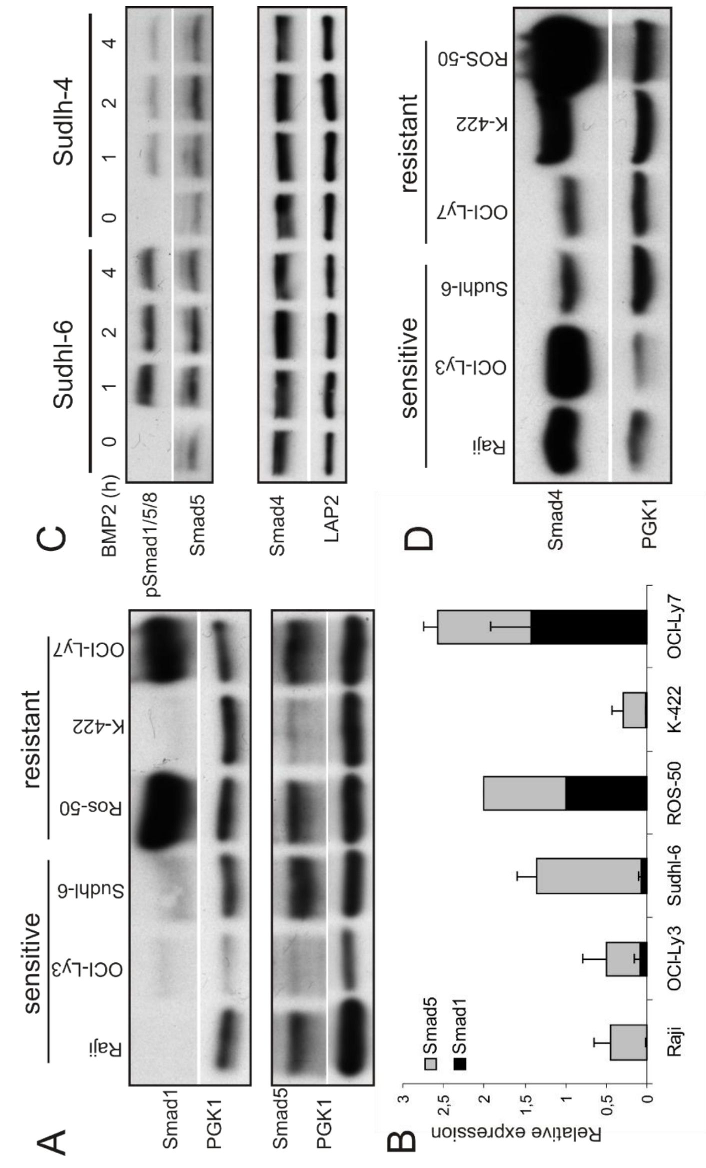
Figure 5


A
sensitive
resistant
Raji
OCI-Ly3
Sudhl-6
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K-422
OCI-Ly7
Smad1
PGK1
Smad5
PGK1
B
Relative expression
Smad5
Smad1
0
0,5
1
1,5
2
2,5
3
Raji
OCI-Ly3
Sudhl-6
ROS-50
K-422
OCI-Ly7
C
Sudhl-6
Sudhl-4
BMP2 (h)
0
1
2
4
pSmad1/5/8
Smad5
Smad4
LAP2
D
sensitive
resistant
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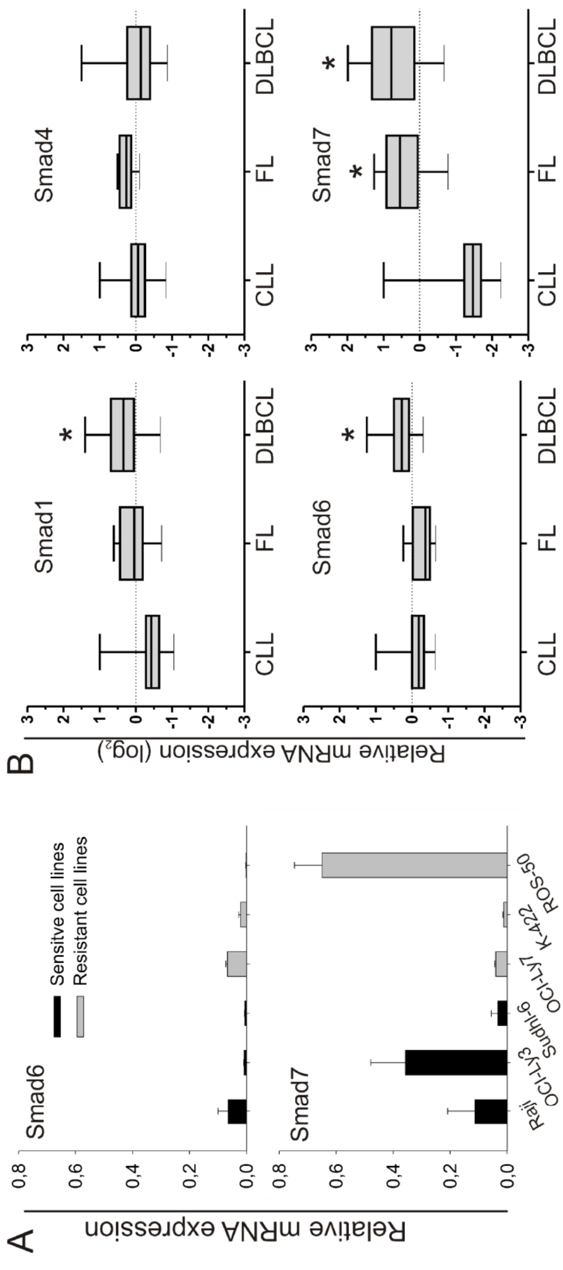
OCI-Ly7
K-422
ROS-50
Smad4
PGK1

Figure 6


A
Relative mRNA expression
Smad6
Smad7
Sensitive cell lines
Resistant cell lines
Raji
OCI-Ly3
Sudhl-6
OCI-Ly7
K-422
ROS-50
B
Relative mRNA expression (log2)
Smad1
Smad4
Smad6
Smad7
CLL
FL
DLBCL

Supplementary to paper III

Supplementary figure legends

Supplementary Figure S1.

BMP-4 mRNA expression in B-cell lymphoma cell lines, determined by real-time RT-PCR. Data are given relative to the expression of BMP in human fetal brain. (Means ± SEM, n = 3)

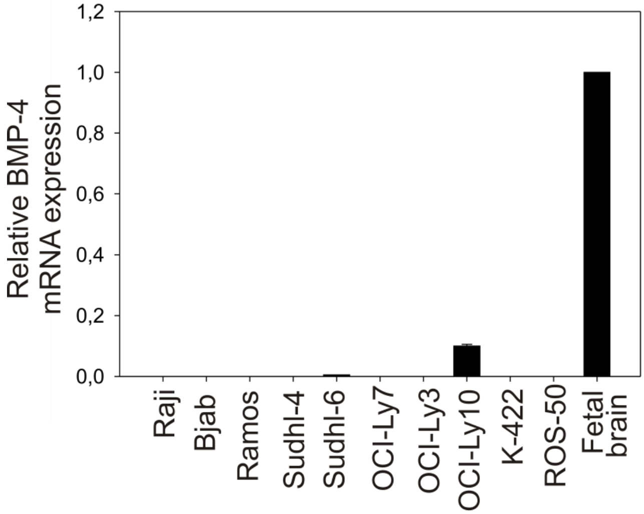
Supplementary Figure S2.

Inhibition of DNA-synthesis in cell lines showing intermediate sensitivity to BMPs. Lymphoma cell lines stimulated with or without BMPs for three days before 3H-thymidine incorporation was measured. Values are obtained by normalizing mean cpm for each BMP to the mean cpm for unstimulated control in each experiment. (Means ± SEM, n = 6 – 7) * $p < 0.05$.

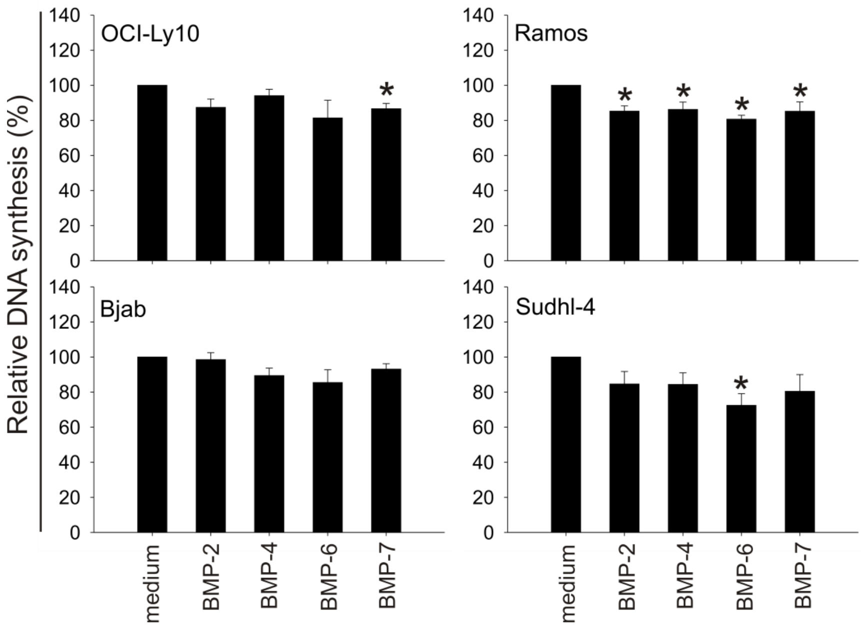
Supplementary Figure S3.

BMP-2, -4 and -6 induce phosphorylation of Smad1/5/8 that is selectively blocked by Dorsomorphin in sensitive Sudhl-6 cells. A, cells were cultured with BMP-2, -4 or -6 for different periods of time, and analyzed for pSmad1/5/8 expression by Western blotting. B, cells stimulated with various BMPs or TGF-β with or without Dorsomorphin for one hour before cells were lysed to detect pSmad/1/5/8 induction. C, cells were stimulated with or without various BMPs or TGF-β, in the presence or absence of Dorsomorphin for three days before 3H-thymidine incorporation was measured. (Mean cpm values ± SEM, n = 6, (n = 2 for Dorsomorphin only)).

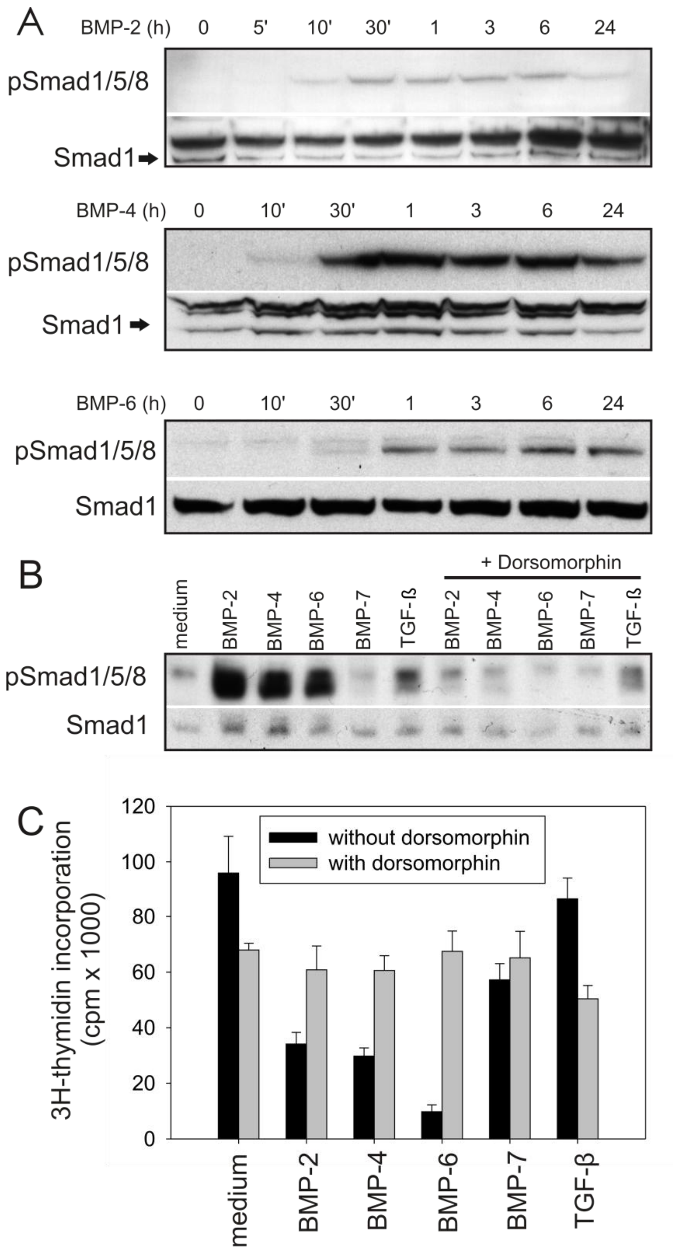
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Table I: Analysis of BMP-induced cell death in lymphoma cell lines[a]

		% PI positive cells				
	Cell line	medium	BMP-2	BMP-4	BMP-6	BMP-7
Resistant	ROS-50	5 ± 1	4 ± 1	4 ± 1	5 ± 1	5 ± 1
	K-422	6 ± 2	5 ± 1	5 ± 1	7 ± 2	5 ± 1
	OCI-Ly7	8 ± 2	9 ± 2	10 ± 3	12 ± 4	10 ± 4
Reduced sensitivity	Bjab	15 ± 4	15 ± 4	19 ± 4	17 ± 4	12 ± 3
	Ramos	4 ± 0	5 ± 1	5 ± 1	7 ± 1	6 ± 1
	OCI-Ly10	21 ± 1	21 ± 1	20 ± 2	25 ± 2	22 1
	Sudhl-4	3 ± 1	4 ± 1	4 ± 1	4 ± 1	4 ± 1
Sensitive	OCI-Ly3	21 ± 2	21 ± 2	20 ± 1	20 ± 2	19 ± 1
	Raji	5 ± 1	6 ± 1	7 ± 1	5 ± 1	5 ± 1
	Sudhl-6	6 ± 1	15* ± 2	16* ± 1	31* ± 3	8* ± 3

[a]Lymphoma cell lines were stimulated with or without BMPs for 3 days before PI-positive cells were detected by FACS analysis. Means ± SEM, n = 6-7, * $p < 0.05$

Supplementary Table II: BMP-2 and -6 induce apoptosis in Sudhl-6[a]

	% TUNEL positive cells		
	medium	BMP-2	BMP-6
Day 1	18 ± 9	32 ± 7	35 ± 4
Day 2	11 ± 5	39 ± 4	60 ± 2
Day 3	11 ± 3	39 ± 7	72 ± 3

[a]Sudhl-6 cells were stimulated with BMPs for 3 days before apoptosis was quantified with TUNEL. Mean values ± SEM are shown (n = 3).

Supplementary Table III: BMP receptor expression in tumor B cells and in normal infiltrating B cells from FL patient lymph node specimens

		Relative MFI					
patient	B cell subset	Alk2	Alk3	Alk6	BMPRII	ActRIIA	ActRIIB
FL1	Normal	2.9	1.9	2.6	1.5	1.9	2.2
	Tumor	7.8	1.7	2.5	1.2	2.0	3.1
FL2	Tumor	4.9	nd[a]	2.5	1.9	3.8	5.0
	Normal	3.6	nd[a]	1.6	1.4	1.5	6.3
FL5	Tumor	4.5	2.5	4.3	2.4	2.3	7.4
	Normal	1.5	1.1	1.2	1.0	1.0	5.0
FL6	Tumor	3.9	1.6	1.9	1.2	1.4	5.0
	Normal	2.3	1.3	1.9	0.9	1.1	6.6

[a] nd = not determined